Summer 2017

The effect of calcium in artificial substrates for oyster restoration: Implications for the mitigation of oyster population decline

Jessie Mandirola
James Madison University

The Effect of Calcium in Artificial Substrates for Oyster Restoration: Implications for the Mitigation of Oyster Population Decline

Jessie Mandirola

A thesis submitted to the Graduate Faculty of

JAMES MADISON UNIVERSITY

In

Partial Fulfillment of the Requirements

for the degree of

Master of Science

Department of Biology

August 2017

FACULTY COMMITTEE:

Committee Chair: Dr. Patrice Ludwig

Committee Members/ Readers:

Dr. Christine May

Dr. Louie Wurch

Acknowledgments

I would like to first acknowledge my advisor Dr. Patrice Ludwig. She has been instrumental in making me a better student, biologist, and person. I would not have been able to complete this thesis without her unwavering support and her ability to brighten my days over the past two years. Next, I would like to thank my committee members Dr. Christine May and Dr. Louie Wurch. Dr. May has been a wonderful companion to turn to and her unending knowledge has been a gift. Dr. Wurch was the driving force behind the second-half of this thesis. I would not be able to have this well-rounded thesis without his knowledge, equipment, and undergraduate students. I would also like to acknowledge the Tidewater Oyster Gardening Association for their collaboration with James Madison University. It was through that relationship I built that I was able to use their property as my field site for the past two years as well as their knowledge of oyster restoration. Next, I would like to acknowledge the James Madison University Biology Department for all of their support including providing transportation to my field site and supporting my travel to conferences to share my research. In addition, I would like to acknowledge my fellow graduate students, many of whom assisted me in my research. They have been an unending source of encouragement, support, and laughter. Finally, I would like to thank my family who has been there for me through it all. Even though they have been a few states away over the past two years, they never stopped showing how proud they are. Thank you all so much!

Table of Contents

List of Tables

List of Figures

Abstract

Oyster populations in the Chesapeake Bay have declined by 99% over the past 150 years due to overharvesting, disease, ocean acidification, and poor water quality. Restoration efforts are needed to reestablish native oyster populations. Current restoration efforts utilize natural oyster shells but these methods are expensive and unsustainable. Therefore, restoration is starting to use artificial substrates instead. Concrete has been successfully used in previous research; spat will attach and oysters will grow. However, there is a lack of knowledge about how the composition of concrete effects oyster larval recruitment. We tested concrete made with limestone sand ("special concrete") to increase the concentration of calcium to better mimic natural oyster shells. After deploying special concrete substrates along with normal concrete and natural oyster shells, we found that oyster larvae preferred to settle on natural shells (Chi-square; $p < 0.001$) but larvae showed no preference between the two concrete types. Over time, natural shells lost more than twice as many larvae than the concrete substrates.

In addition, it must be ensured that artificial substrates being used for restoration are not causing harm to the environment. There is currently no research on how artificial substrates affect localized water quality or algal growth, which is at the base of the food chain on oyster reefs. We tested the effects of these same substrates on water quality (pH, conductivity, alkalinity, calcium) and algal growth (fluorescence and Fv/Fm (difference between minimum and maximum fluorescence)). The special concrete substrate caused lower pH and alkalinity levels, but their values were within normal ranges and were likely not biologically significant. In addition, the special concrete substrate showed minimal effect on the growth of several algal strains.

Both concrete substrates were successful in larval recruitment and showed no negative effects on localized water quality or algal growth. Therefore, we can conclude that the use of an artificial substrate with augmented levels of calcium to better mimic natural oyster shells can be used as a restoration substrate to help reestablish oyster populations. Long-term studies do need to be conducted to determine the lasting effects of these substrates as oyster restoration tools.

I. Introduction

Introduction

Habitat Selection

Habitat use and habitat selection are common ecological concepts that are, at times, erroneously interchanged. Therefore, it is important to distinguish between "habitat use" versus "habitat selection". Habitat use refers to the way in which an organism uses its surroundings to fulfill life history milestones (Block and Brennan 1993). Habitat selection, however, is an organism's behavioral response to particular characteristics of a habitat and the ability of that habitat to increase their lifetime fitness (Hutto 1985, Block and Brennan 1993). This behavior implies that organisms are able to recognize and assess fitness costs and/or benefits of different habitats (Jones 2001). When an organism displays habitat selection behaviors, it typically leads to disproportionate settlement in habitats with varying resource levels. These differences in resource levels translate to a difference in fitness levels (Block and Brennaan 1993).

Most early research done on habitat selection involved bird species (Kendeigh 1945, Sväardson 1949, Hutto 1985, Block and Brennan 1993, Donzár et al. 1993), but, in recent years, other land-dwelling animals have been of interest (Beebee 1983, Mauritzen et al. 2003, Cairniello et al. 2007, Land et al. 2008), as well as aquatic organisms (Meadows and Campbell 1972, Morrissey and Gruber 1993, Hastie et al. 2003). For both land-dwelling and aquatic organisms, habitat selection is based on the subsequent reproductive success that a specific habitat can support. However, the way in which mobile and sessile aquatic organisms choose their habitat is fundamentally different because of the differences in their ability to relocate once they settle.

Mobile marine organisms have control over where they settle because they can move if resources become scarce. In fact, many marine animals are migratory and follow a global food cycle. However, the dispersive juvenile stage of many sessile species have much less control over where they settle, which has consequences in later life stages. For example, sessile marine species are unable to move to seek and then reproduce with a mate, if mates are scarce. Natural selection has shaped a reproductive strategy of releasing millions of egg and sperm directly into the water column. Gametes combine to create drifting or free-swimming larvae that must eventually settle in a favorable habitat in order to reproduce in the future. However, the larvae have to do this with little knowledge of the environment they are in. Sessile marine species use conspecifics as a reliable settlement cue because it signals a favorable habitat for survival and eventual reproduction (Raimondi 1988, Lecchini et al. 2010, Lecchini et al. 2017). For example, Raimondi (1988) was able to induce recruitment and settlement of the barnacle *Chthamalus anisopoma* outside its typical vertical range by using an adult conspecific extract applied to a settling surface. However, this is just one example of a cue. The mechanisms behind sessile organism habitat selection at the larval stage are more complicated. Habitat selection can be based on chemical cues from adult conspecifics (Burke 1983, Coon and Bonar 1985, Raimondi 1988, Bonar et al. 1990, Turner et al. 1994, Zimmer-Faust and Tamburri 1994, Lecchini et al. 2010, Lecchini et al. 2017), water flow (Turner et al. 1994), water temperature (Nair and Appukuttan 2003), biofilm production on the settling substrate (Bonar 1990, Faimali et al. 2003), and/or olfactory cues (Dixson et al. 2011).

There are a two main ways in which habitat selection is studied in sessile marine species. Most experiments are done in the laboratory with controlled selection scenarios with specific variables being carefully manipulated (Bonar et al. 1990, Turner et al. 1994, Nair and Appukuttan 2003, Faimali at el. 2004). Less research has been done on habitat selection of sessile marine organisms in natural environments due to the difficulty of determining the mechanism, not just the phenomenon of habitat selection. However, researchers have been able to combine laboratory and field experiments to determine some mechanisms behind oyster larval habitat selection as reviewed below.

Habitat Selection in Oysters

Two main pathways of habitat selection have been determined for oyster larvae: (1) chemical cues given off by adult and juvenile conspecifics, (2) substrate type and biofilm production on the substrate. Oyster larvae are triggered by a stimulus in the surrounding environment to settle and those stimuli are only present in areas with conspecific adults and juveniles present (Cole and Knight-Jones 1949, Crisp 1967, Hidu 1969, Burke 1983). In laboratory experiments, an amino acid, L-3,4-dihydroxyphenylalanine, (L-DOPA, subsequently converts to dopamine) was artificially applied to controlled aquaria containing oyster larvae (Bonar et al. 1990) in order to stimulate settling behavior (alternating between swimming and crawling on a substrate). When a blocking agent was applied to the water to prevent the amino acid from interacting with the larvae, the typical settling behavior was not present (Bonar et al. 1990). This lack of response suggests that L-DOPA is a settling signal that juvenile oysters use to signal the presence of conspecific adults. In addition, substrate type, along

with biofilm production on substrates, has been used to determine habitat selection preferences in oyster larvae (Soniat et al. 1991, Soniat and Burton 2005, Tamburri et al. 2008). When given the choice between conspecific oyster shells and non-natural substrates like fiberglass and granite in a laboratory-controlled experiment, oyster larvae preferred to settle on the natural substrate (oyster shell) more often than the non-natural substrate (Tamburri et al. 2008) measured by the percent settled spat on each of the substrates. In field experiments, oyster larvae were given a choice between limestone containing high concentrations of calcium, which mimics natural oyster shells, and sandstone containing little to no calcium (Soniat and Burton 2005). The larvae preferred to settle on the calcium-rich limestone more often than the calcium-poor sandstone, suggesting the chemical makeup of the substrate influences larval habitat preference as well.

The combinations of adult chemical cues and substrate type clearly have an influence in where oyster larvae eventually settle for reproduction. However, oyster populations across the globe have declined by 85% in the past 200 years (Zu Ermgassen et al. 2012). With this drastic decline, oyster larvae are no longer receiving chemical cues from adult conspecifics and are unable to settle on favorable substrates known to support large populations of oysters. In addition, even using natural oyster shells and their biofilm alone for restoration is unsustainable because of the limited supply of shells (Mann and Powell 2007). Therefore, optimizing restoration efforts using artificial substrates is needed to allow for oyster larvae to have a favorable environment to develop.

Use of Artificial Substrates for Restoration

The Chesapeake Bay was one of the most productive oyster-harvesting sites in the world. However, only about 1% of the native oyster population remains (Beck et al. 2011, Dunn et al. 2014). Current restoration efforts in Virginia are not sustainable enough to last into the future (Mann and Powell 2007). Natural shells are becoming increasingly expensive because they are rare and the current restoration efforts are not efficient enough to bring oyster populations back to their historical quantities. Novel approaches are needed to make sure that oyster reefs are maintained as vital components of the intertidal zones of the Chesapeake Bay. Recently, there has been a shift towards using artificial substrates to augment and create new oyster reefs (Tamburri et al. 2008, Whitman and Reidenbach 2012, Drexler et al. 2014, Dunn et al. 2014, Theuerkauf et al. 2015). Artificial substrates have been used in previous studies to look at the settlement and survival of barnacles (Caffey 1982). Caffey (1982) found that barnacle larvae settled on several different rock types (shale, sandstone, mudstone, and gabbro) with no specific preference. This result suggests that these invertebrates are capable of settling on artificial substrates and leads to the question of if other invertebrates are capable of settling on artificial substrates. The goal of discovering adequate artificial substrates to replace natural elements for restoration is to reduce the overall cost of restoration efforts, which will allow for a wider adoption of management techniques in Virginia.

Several substrates have been studied to determine their effectiveness of larval oyster recruitment and subsequent larval survival (Soniat et al. 1991, Soniat and Burton 2005, Drexler et al. 2014, Dunn et al. 2014, Theuerkauf et al. 2015). Materials such as limestone, gravel, marl (calcium carbonate-based material containing clay and aragonite),

granite, and concrete have all been studied because they are affordable, do not decompose at a fast rate, and would be an acceptable choice for the settlement of oyster larvae (Soniat et al. 1991). It takes up to three years to create a viable oyster reef (Bahr and Lanier 1981), therefore, the substrates chosen need to be able to last long enough for a new oyster reef to develop to a point where substrate support is no longer required. Even though all of the substrates listed conform to these guidelines, concrete is the most widely available, the least expensive, and most malleable. Therefore, concrete is the basis of most studies looking at the success of artificial substrates to augment natural oyster reefs.

Previous studies by Theuerkauf et al. (2015), Dunn et al. (2014), and Drexler et al. (2014) found that concrete is a suitable alternative substrate for recruitment and subsequent survival of oyster larvae when compared to natural shell. Theuerkauf et al. (2015) used concrete Oyster Castles® to compare recruitment success with natural oyster shells and vertically embedded oyster shells into concrete (Figure 1.1). They found that Oyster Castles® could recruit a similar density of oyster larvae as natural oyster shell. Total recruit density on Oyster Castles® and natural oyster shell (88 larvaem^2, 80 larvae/m^2, respectively) was three times higher than on the embedded oyster shells (28 larvae/m^2; $p < 0.05$; Theuerkauf et al. 2015). They also found that Oyster Castles® supported a higher survival rate of recruited larvae when compared to natural oyster shell and embedded oyster shell (91%, 60%, 60%, respectively, $p < 0.05$; Theuerkauf et al. 2015). They concluded that Oyster Castles® provided enough vertical relief and interstitial space to attract and support a similar, if not greater, amount of oyster larvae as natural shells.

Figure 1.1. Oyster Castles Oyster Castles® are successful as an oyster restoration tool in many estuarine habitats. Adapted from the U.S. Fish and Wildlife Service- Building Living Reefs in Southern New Jersey webpage.

Dunn et al. (2014) also used concrete, as well as marl and granite, to compare initial larval settlement and shell growth to natural oyster shells. It was found that concrete and natural shells had similar recruitment densities (~700 oysters/m^2, $p = 0.053$), which were higher than densities on both marl and granite (~100 oysters/m^2, ~200 oysters/m^2).

Drexler et al. (2014) looked at the differences in larval recruitment of oysters using manmade seawalls made of concrete as a reef base versus natural reefs. They found that oysters on seawalls had similar recruitment rate success to oysters on natural reefs during peak recruitment time (July; ~14 spat/shell, $p = 0.75$). They also found that the mean biomass of larvae was the highest on the seawall (507 g/m^2), concluding that oysters using manmade seawalls as reef substrates were just as successful at recruitment as oysters at a natural reef system.

These three studies clearly demonstrate that concrete is a suitable artificial substrate that can support the creation of new oyster reefs for restoration purposes. In addition, they demonstrate that oyster larvae can choose to settle on artificial substrates

just as they choose to settle on natural oyster shells. The continued use of artificial substrates for oyster restoration can help supplement the reduced number of oyster populations in the Chesapeake Bay. What has not been studied however, is how different chemical formulations of concrete being used for oyster restoration could affect larval settlement as well as the local water quality and algae growth as the base of the food web.

Unexplored Methods

After several generations of spawning and settling, oysters are able to create dense, complicated reefs because oyster larvae settle on/near adult conspecifics (Figure 1.2). To date, studies have only addressed whether concrete is a suitable substrate to attract similar numbers of larvae as natural oyster shells during the recruitment period for restoration purposes. What have not been explored are modifications that could be made to concrete to optimize recruitment and longevity. The next wave of artificial reef research should focus on questions of optimization, not efficacy, of concrete. Additionally, community effects of concrete substrates should also be of focus.

Figure 1.2 Comlex Oyster Reef. Generations of oyster larvae settling on top of each other leads to complex reef systems. Adapted from James Cook University- Tropical Water & Aquatic Ecosystem Research webpage.

Oyster Reefs as Habitat

Several studies show that these unique oyster reefs create habitats that allow for a diverse array of vertebrate and invertebrate organisms that directly benefit from the oysters complex manufacturing of reefs (Bahr and Lanier 1981, Peterson et al. 2003, Grabowski and Peterson 2007). For example, various species of fish, like gobies and seatrout, and invertebrates, like polychaetes and crustaceans, often use oyster reefs as foraging grounds and refuge from predators or during periods of high-energy wave action (Boudreaux et al. 2006, Coen et al. 2007, Grabowski and Peterson 2007, Soniat et al. 2007). Coen and Luckenbach (2000) found that there was higher cumulative species richness over time found on oyster reefs when compared to clam reefs. They also found that the mean number of species found per reef type (oyster, clam) was consistently higher on the oyster reef over a one-year sampling period (Coen and Luckenbach 2000).

The food web, and water quality, associated with manufactured oyster reef systems could be directly affected by the use of artificial substrates like concrete. Several species rely on the existence of oyster reefs for protection from predation and high-energy waves as well as for food sources like algae. As for any restoration effort, we must ensure that we are not harming the environment when using artificial substrates. Restoration efforts need to take into consideration how artificially engineering oyster reef systems could impact bottom-up food web interactions as well as oyster larval settlement due to their ability to choose their lifetime habitat.

The research presented here aims to explore the use of a new concrete formulation as an artificial substrate for oyster restoration and how that new formulation effects localized water quality as well as subsequent algal growth. Combining these three elements of larval recruitment success, effect on localized water quality, and the subsequent effect on localized algal growth, we build a well-rounded argument for how successful artificial substrates for oyster restoration efforts in the Chesapeake Bay can be. This outcome would reduce the reliance on natural shells or potentially harmful alternative substrates to restore oyster reefs.

II. Larval Settlement on Varying Substrates

Abstract

The high national priority of developing artificial oyster reef substrates is a consequence of scarcity and expense of natural oyster shells available for restoration. Restoration is imperative because the historical abundance of native oyster populations has declined 99% over the past 100 years. A common artificial reef substrate is concrete made with silica sand. The aim of this research is to test alternative concrete formulations in oyster restoration efforts. Specifically, we tested the hypothesis that wild juvenile oysters would strike to concrete made with limestone sand more often than striking to concrete with silica sand. Limestone sand has elevated levels of calcium, which is known to attract oyster larvae for settlement and growth. Natural oyster shells were used as a control. Each substrate was placed in mesh bags (80 shells and four mesh bags per substrate type) and hung off a dock at Pitman Cove in Kilmarnock, VA. Every two weeks for 14 weeks, the number of settled oyster larvae was recorded. A Kruskal-Wallis post hoc test suggests that at the end of one spawning and recruitment period (14 weeks, 2016), shell casts made with limestone sand attracted similar abundances of oyster spat (settled oyster larvae; $n = 149$) as normal concrete casts ($n = 174$). However, both shell cast varieties attracted fewer spat than natural oyster shells ($n = 365$; Kruskal-Wallis $p < 0.001$). In addition, there was an eventual decline in settled spat across all three treatments. Even though there was no statistical significance in percent decline between treatments (ANOVA; $p = 0.88$), the natural shell lost more than twice as many spat compared to the concrete treatments. The long-term effects of the limestone-rich concrete formulation need to be examined, however, this study suggests that concrete

with augmented levels of calcium do not have initial adverse effects in attracting oyster larvae. We suggest that future restoration efforts should consider using alternative substrates that better mimic natural oyster shells with respect to calcium levels to reduce overall cost while maintaining optimal recruitment success.

Introduction

Oysters have critical ecological benefits including filter feeding (Drexler et al. 2013), assimilating nitrogen and phosphorus (Kellogg et al. 2013), creating living shorelines (Theuerkauf et al. 2015), and supporting high biodiversity (Peterson et al. 2003). Their economic benefits include supporting recreational and commercial fisheries for hundreds of years (Drexler et al. 2013). However, due to overharvesting (Woods et al. 2005), disease (Cook et al. 1998), poor water quality (Gottlieb and Schweighofer 1996), and ocean acidification (Kemp et al 2005), global oyster populations have declined by 85% (Beck et al. 2011, Zu Ermgassen et al. 2012). In the Chesapeake Bay, the native oyster population, *Crassostrea virginica*, stands at less than 1% of its historical quantity (Dunn et al. 2014).

Within the past 50 years, several restoration efforts have been employed in the Chesapeake Bay to help slow the rapid population decline and restore the natural benefits that oysters provide (Grabowski and Peterson 2007, Schulte et al. 2009, Schulte and Burke 2014). There are currently several methods used to create and augment oyster reefs in the Chesapeake Bay according to the National Estuaries Restoration Inventory (NERI, https://neri.noaa.gov/neri/), all of which use natural shell elements as the basis of the reconstruction: (1) stock enhancement, (2) oyster gardening, and (3) deployment of

natural materials (Brumbaugh and Coen 2009). However successful these methods are, none of them can be maintained as a viable restoration option in the future because of the limited availability of natural shells (Brumbaugh and Coen 2009). New approaches are needed to ensure that oyster reefs are maintained as vital components of the intertidal zones of the Chesapeake Bay.

Recently, restoration efforts have shifted towards using artificial substrates to augment and create new oyster reefs in the Chesapeake Bay (Theuerkauf et al. 2015, Dunn et al. 2014, Whitman and Reidenbach 2012, Drexler et al. 2014, Tamburri et al. 2008). Concrete has been the most widely used and most successful artificial substrate used for oyster restoration due to its success in recruiting oyster larvae, relatively low cost, and its ability to be shaped as needed (Soniat et al. 1991), typically into simple shapes such as oyster reef balls or Oyster Castles® (Figure 2.1). What has not been addressed with these studies, however, is the chemical composition of the concrete being used and the short-term and long-term impacts of the chemical makeup of the concrete.

Figure 2.1. Artificial Substrates Oyster reef balls (left) and Oyster Castles® (right) are widely used as artificial oyster habitats for restoration efforts. Adapted from the (left) Reef Innovations and (right) U.S. Fish and Wildlife Service- Building Living Reefs in Southern New Jersey webpages.

Oyster larvae are able to choose their settlement location based on several physical and biological properties (Bonar et al. 1990). Among these properties is biofilm production on the substrate surface (Faimali et al. 2004, Crisp 1967, Tamburri et al. 1992) and chemical signals from the surrounding environment and adult conspecifics resulting in gregariousness (Hidu 1969, Veitch and Hidu 1971, Bahr and Lanier 1981, Burke 1986, Burke 1986, Soniat et al. 1991, Soniat and Burton 2005, Tamburri et al. 2007). Several molecules have been shown to influence oyster settlement including amino acids, ammonia, and calcium (Hidu at el. 1975, Coon and Bonar 1985, Bonar et al. 1990, Soniat et al. 1991, Turner et al. 1994, Zimmer-Faust and Tamburri 1994, Soniat and Burton 2005). More recently, the presence of calcium in settling substrates has been explored as a possible element of success for artificial substrates due to its presence in natural oyster shells. Soniat and Burton (2005) examined the effectiveness of limestone and sandstone on spat (settled oyster larvae) recruitment. They found that spat had a clear preference for limestone suggesting that the presence of calcium in the limestone substrate was more attractive to the spat than the quartzitic sandstone. In addition, Tamburri et al. (2008) explored the relationship between substrate types (*C. virginica* shell, granite, fiberglass, PVC, stainless steel), biofilm production, and larval settlement in *C. virginica.* They found that spat preferred to settle on conspecific shells containing calcium that had a well-established biofilm compared to any of the substrates without a well-established biofilm. Biofilm production on the surface of a substrate is a factor of the chemical makeup of the substrate (Faimali et al. 2004) suggesting that larvae are able to detect the calcium in the substrate as well as in the biofilm on the surface of the

substrate. These results reinforce that calcium is a major influencing factor in attracting larvae for settlement and growth.

Manipulative experiments on the chemical composition of alternative substrates have not been done. The purpose of this study is to determine the spat recruitment success on an alternative oyster restoration substrate that contains elevated levels of calcium and an established biofilm. While cement is made of 60-67% lime (CaO), it is mixed with silica sand, thus reducing the overall calcium levels in concrete restoration substrates. Substituting silica sand with an additional source of calcium carbonate (limestone sand) could make artificial reef structures even more attractive to spat compared to traditional concrete and, thus, speed restoration efforts. In addition, we used normal concrete shell casts (made with silica sand) and natural oyster shells as reference substrates to gauge the success of the augmented shell casts, hereafter referred to as special concrete shells. Natural oyster shells are made up of ~ 90% calcium (Yoon et al. 2003, Hamester et al. 2012) making it the substrate with the highest level of calcium (Table 2.1). By adding limestone sand instead of silica sand, we effectively doubled the level of calcium compared to the normal concrete shells. Therefore, the 'special' concrete shells had the next highest level of calcium followed by the normal concrete shells (Table 2.1).

Table 2.1. Calcium Levels The substrates used in this study had varying levels of calcium relative to each other based on the way they were made.

Substrate	Calcium Level
Natural Shell	High
Special Concrete	Intermediate
Normal Concrete	Low

We hypothesize that spat recruitment will be greater on the special concrete than on the normal concrete shell casts. We also hypothesize that the natural shell will have the greatest spat recruitment. By combining the success of concrete as an alternative substrate and the chemical composition of natural oyster shells, we hope to discover the use of a new, inexpensive, malleable, substrate to be used for future oyster restoration.

Materials and Methods

Study Organism

Classification. Oysters are part of the class Bivalvia, which includes mussels, clams, and scallops, and are characterized by a shell divided into 2 valves, a laterally compressed body, and a large mantle cavity that holds gills (Bahr and Lanier 1981). The eastern oyster, *Crassostrea virginica,* is the oyster of interest in this study. *C. virginica* is characterized by several features: extremely variable shell shape at maturity, paired gills, a distinctly asymmetrical promyal chamber that allows for a greater pumping rate and a higher release velocity of gametes for dispersal, ability to tolerate wide ranges of salinity, temperature, and turbidity, and a wide natural range along the east coast from Canada to Florida (Bahr and Lanier 1981).

Reproduction. Oysters are dioecious, having distinct male and female individuals (Bahr and Lanier 1981). Males tend to fully develop within their first year whereas females develop within 2 years (Bahr and Lanier 1981). After male maturity, a drastic temperature or salinity change triggers males to release sperm into the water column (Bahr and Lanier 1981). A protein pheromone in the sperm then triggers the females to

release their eggs. The relationship between the male release of sperm and subsequent release of eggs by females can cause a large chain reaction across a dense population of oysters (Bahr and Lanier 1981).

After the release of sperm and eggs, fertilization occurs in the water column, through chance encounters, and larvae development begins (Bahr and Lanier 1981; Figure 2.2). While in the water column, they develop a foot with which they will use to attach to a substrate, as well as a set of darkly pigmented eyes signaling they are ready to undergo transformation into the adult form (Bahr and Lanier 1981; Figure 2.2). Typically, the larval stage lasts between 7 and 10 days, but can last up to 2 months (Bahr and Lanier 1981) depending on the quality of the environment, including water temperature and food availability (algae, protozoa, bacteria, dissolved organic matter).

After about 2 weeks, there are several environmental factors that induce settlement of mature larvae including light, salinity, temperature, and current strength (Bahr and Lanier 1981). In addition to these triggers, it was suggested that larvae respond to chemical cues (ammonium, amino acids, calcium) released by adult oysters as well as the biofilm that develops on the shells of adult oysters (Hidu at el. 1975, Coon and Bonar 1985, Bonar et al. 1990, Soniat et al. 1991, Turner et al. 1994, Zimmer-Faust and Tamburri 1994, Soniat and Burton 2005). This suggests that young, free-swimming oyster larvae are able to choose where they settle and begin development based on the presence/absence of these chemical cues from adult conspecifics.

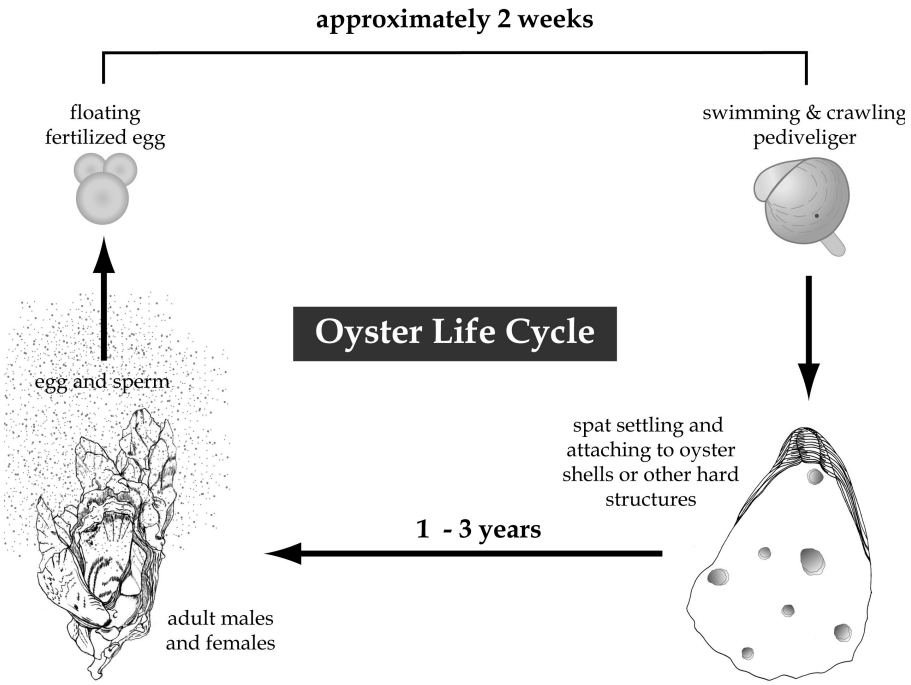

Figure 2.2. Oyster Life Cycle The general life cycle of oysters. Adapted from the South Carolina Oyster Restoration and Enhancement webpage.

Adulthood. Once the larvae settle, they undergo metamorphosis of their internal organs and are referred to as spat. The spat begin to sequester calcium carbonate from the water column to make their shells (Bahr and Lanier 1981). Typically, it takes up to 3 years for a spat to become an adult oyster with average shell growth being up to 25 mm per year (Bahr and Lanier 1981; Figure 2.2). Year after year of spat settling on previous generations of oysters creates dense and intricate oyster reef systems.

Site Description

This fieldwork for this study took place at a field site in Kilmarnock, Virginia between July 11, 2017 and October 23, 2017. Kilmarnock, Virginia is located on the

Northern Neck of the Chesapeake Bay just north of the Rappahannock River (Figure 2.3). Pitman Cove is situated on the YMCA Camp Kekoka property at 37° 41’ 39.4” N and -76° 21’ 09.5” W just west of Grace Point. The cove is an inlet with very little wave action throughout the year due to the protection it receives from surrounding land. The average surface salinity of Pitman Cove ranges from 10-20ppt year round but is typically between 12 and 15ppt (Chesapeake Bay Program-Salinity). Water temperatures vary by season with summer being the warmest (25°C) and winter being the coldest (5°C; Maracoos 2015; data collected 1993-2014).

Historically, the site was used as the Alexandria Police Youth Camp from 1946 to 2007 when the YMCA bought the property to be used as a summer camp. Pitman Cove is currently the site of oyster gardening projects put on by the Tidewater Oyster Gardening Association (TOGA). TOGA is a non-profit organization, established in 1997, that promotes the health of the Chesapeake Bay through oyster cultivation. The members are involved in educating the public about the many benefits that oysters provide as well as the many ways that people can get involved in oyster aquaculture.

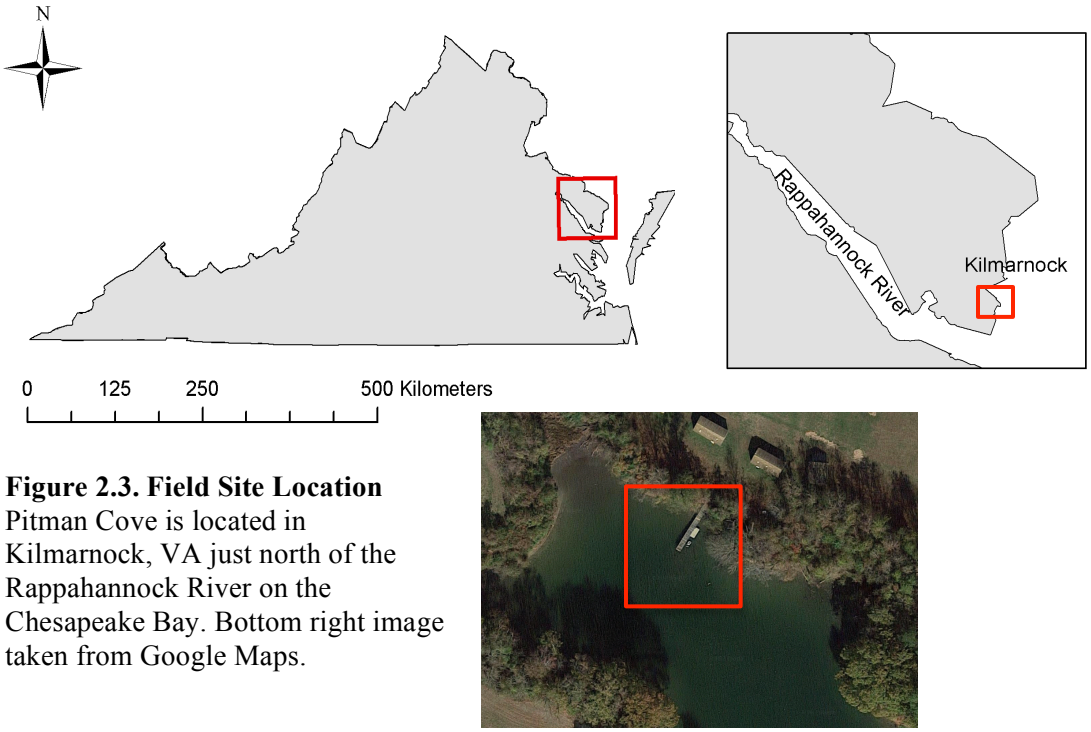

Figure 2.3. Field Site Location
Pitman Cove is located in Kilmarnock, VA just north of the Rappahannock River on the Chesapeake Bay. Bottom right image taken from Google Maps.

Manufacturing of Shells

Manufacturing of Silicon Molds. In order to create the concrete casts (special and normal) molds of natural oyster shells needed to be made (Figure 2.7). The flat side of an oyster shell was pressed into clay with the convex side outside of the clay. The clay was then molded into half of a Solo© Cup such that the convex side of the shell is facing the middle of the Solo© Cup. A 100:10 gram ratio of MoldMax® 30 (silicon mold making material) Part A and MoldMax® 30 Part B were mixed together and poured into the Solo© Cup to top of the oyster shell. The mixture dried overnight and was removed from the Solo© Cup. Next, the clay was removed from the shell such that the flat side was still attached to the convex side. The mold, and shell, was then placed into a new Solo© Cup such that the flat side was facing the middle of the Solo© Cup. Another 100:10 gram ratio mixture of MoldMax® 30 Part A and MoldMax® 30 Part B was poured into the

Solo© Cup to the top of the oyster shell. Again, the mixture dried overnight and was removed from the Solo© Cup. Using a knife, a slit was cut along the side of the mold to the top in order to remove the oyster shell.

Manufacturing of Normal Concrete Shells. Using the molds, we created the concrete casts by measuring a 2:2:1 gram ratio of Portland cement to Quikrete® All-Purpose Sand to DI water (Figure 2.7). The mixture was poured into the silicon mold and let to dry for 24 hours (Figure 2.4).

Manufacturing of Special Concrete Shells. The procedure was repeated for the manufacturing of special concrete shells, but with limestone sand (provided by Frazier Quarry) instead of Quikrete® All-Purpose Sand (Figure 2.4 and 2.7).

Figure 2.4. Manufacturing Concrete Substrates The concrete mixtures (concrete-sand and concrete-limestone) were placed into the silicon molds (top left) and let to dry overnight (top right). Once dry, they were removed from the molds (bottom) and numbered for deployment.

Deployment

Preliminary Field Work. The natural oyster spawning time needed to be established to determine when to deploy the experimental shells (Figure 2.7). Forty units of natural oyster shell were placed into two 1" mesh bags resulting in 20 units per bag. The bags were hung off the edge of a dock until completely submerged (at high tide and low tide) into Pitman Cove, Kilmarnock, VA (37° 41' 39.4" N, -76° 21' 09.5" W; Figure 2.3). At weekly intervals, visual identification of oyster spat on the oyster shells was verified. Once spat settlement was confirmed, experimental shells were deployed on July 11, 2016.

Preparation for Shell Deployment. Each experimental shell was given a unique identifying number (0001 – 0240) on a smooth, easily visible surface with Sharpie in order to track the settlement success of each individual experimental unit. Twelve plastic 1" mesh bags were made using Oyster Netting from Quadel Industries Inc. Eighty special concrete shells were haphazardly placed into four mesh bags resulting in 20 units per bag (Figure 2.7). The process was repeated for the normal concrete and natural oyster shell units. Each bag contained only one substrate type resulting in 240 units in 12 bags. Each bag was then labeled with the type of shell it contained and the bag number for that type of shell (1-4) using laminated paper.

Experimental Field Work. Each mesh bag was hung off a dock in haphazard order until completely submerged (at high tide and low tide; Figure 2.5). Oyster spat settlement data was collected biweekly using the following methods; each experimental unit was taken

out of the bag and rinsed both to make visual identification and count of oyster spat (Figure 2.6; Table A1). Once all shells within one bag were inspected, they were returned to the bag and hung in the same location off the dock. This method was repeated for all 12 bags. In addition, water chemistry measurements (pH, salinity, and temperature) were taken at each data collection period using the Vernier LabQuest® 2 interface and the associated calibrated sensors for pH, salinity, and temperature (Table A2). Spat counts and water quality measurements were collected biweekly from July 27, 2016 until October 23, 2016 when the temperature of the water remained below 25°C, the point at which spat settlement slows (Manoj Nair and Appukuttan 2003).

Figure 2.5. Positioning of Mesh Bags Twelve mesh bags were filled with 20 units of one substrate type (natural shell, normal concrete, special concrete) and deployed in Pitman Cove for 14 weeks. Spat counts on each shell were determined biweekly between July 27, 2016 and October 23, 2016.

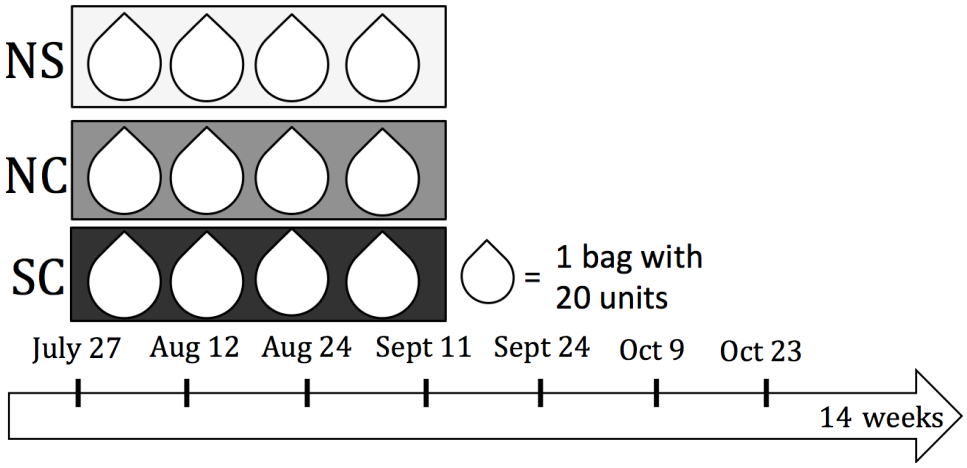

Figure 2.6. **Experimental Design** Spat counts on each experimental unit were collected biweekly for 14 weeks. Specific dates of data collection dates are indicated above. All bags within one treatment (NS = Natural Shell, NC = Normal Concrete, SC = Special Concrete) were pooled for statistical analysis, denoted by the shading of the boxes, due to non-normal distributions. Kruskal-Wallis was performed to test for the statistical difference in spat settlement between treatments at each individual collection period.

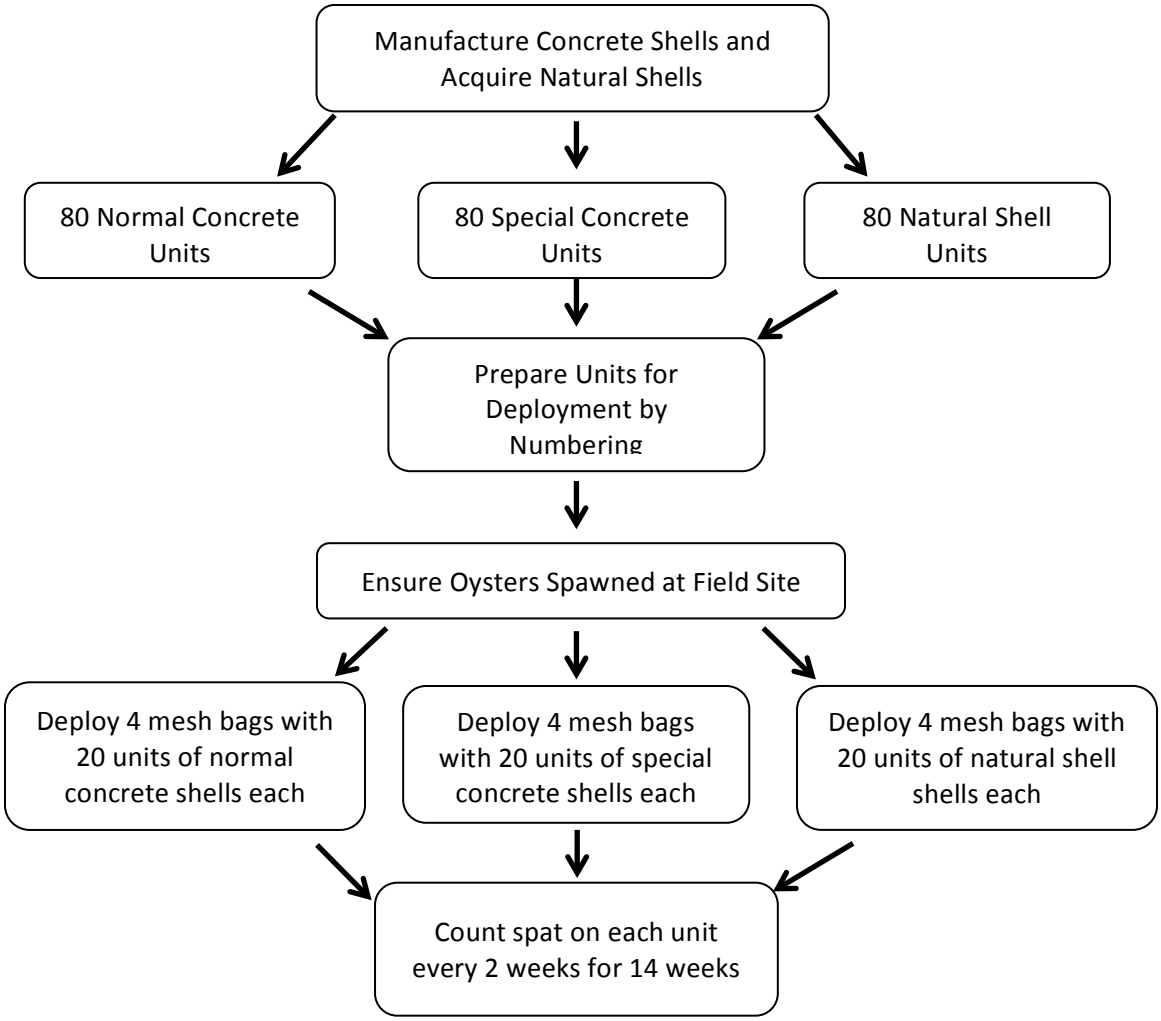

Figure 2.7. Experimental Flow The general flow of the experimental design that took place in 2015 and 2016. More detail is available in the materials and methods.

Statistical Methods

The purpose of this study was to determine the success of three substrate types for oyster restoration measured by the abundance of settled oyster larvae over time, larval preference for a particular settling substrate, and how the abundance on each of the substrates changed.

Settling Preference and Abundance. A Chi-square test was performed to determine if there was a preference of treatment type by the spat on the final data collection date.

For settling abundance, each data collection period was analyzed separately from each other to observe the pattern of settlement and removal of spat on each of the substrate types. The distribution of spat count in each bag (n=12) failed the Shapiro-Wilk test for normality ($p < 0.05$) suggesting skewed data and, thus, the need for a non-parametric test for statistical analysis. Kruskal-Wallis was used to test for the statistical difference in total spat counts for each shell treatment at each separate collection period. Bags with the same shell treatment were grouped together for this analysis due to similar skews. A repeated measures analysis was not used because it would have been more appropriate for the analysis of long-term survival of the oysters on each of the substrates. We are more interested in the recruitment success of the substrates at each individual collection time. A Nemenyi test was used for post-hoc comparisons.

Change in Abundance. Finally, the percent increase or decrease between each collection period for each treatment was determined. The percent decrease for the final four collection periods was compared between treatment types. Due to normal distributions (Shapiro-Wilk; $p > 0.05$) and the assumption that enough time had passed between collection times that percent decrease measurements were independent, an ANOVA was used to test for a statistical difference between the percent declines for each treatment type with a Tukey HSD test for post-hoc analysis. All statistical analyses were completed in RStudio (version 1.0.136; Appendix II).

Results

The distribution of spat present on the different treatments greatly varied within collection dates (Figure 2.8). The interquartile range (IQR) in the number of spat present on the special concrete throughout the study period (IQR = 2; Figure 2.9) and normal concrete (IQR = 3; Figure 2.9) was consistently lower than the IQR of spat present on the natural shell treatment (IQR = 5; Figure 2.9). However, the maximum number of spat on each treatment was similar throughout the study period (SC = 16, NC = 14, NS = 18; Figure 2.9).

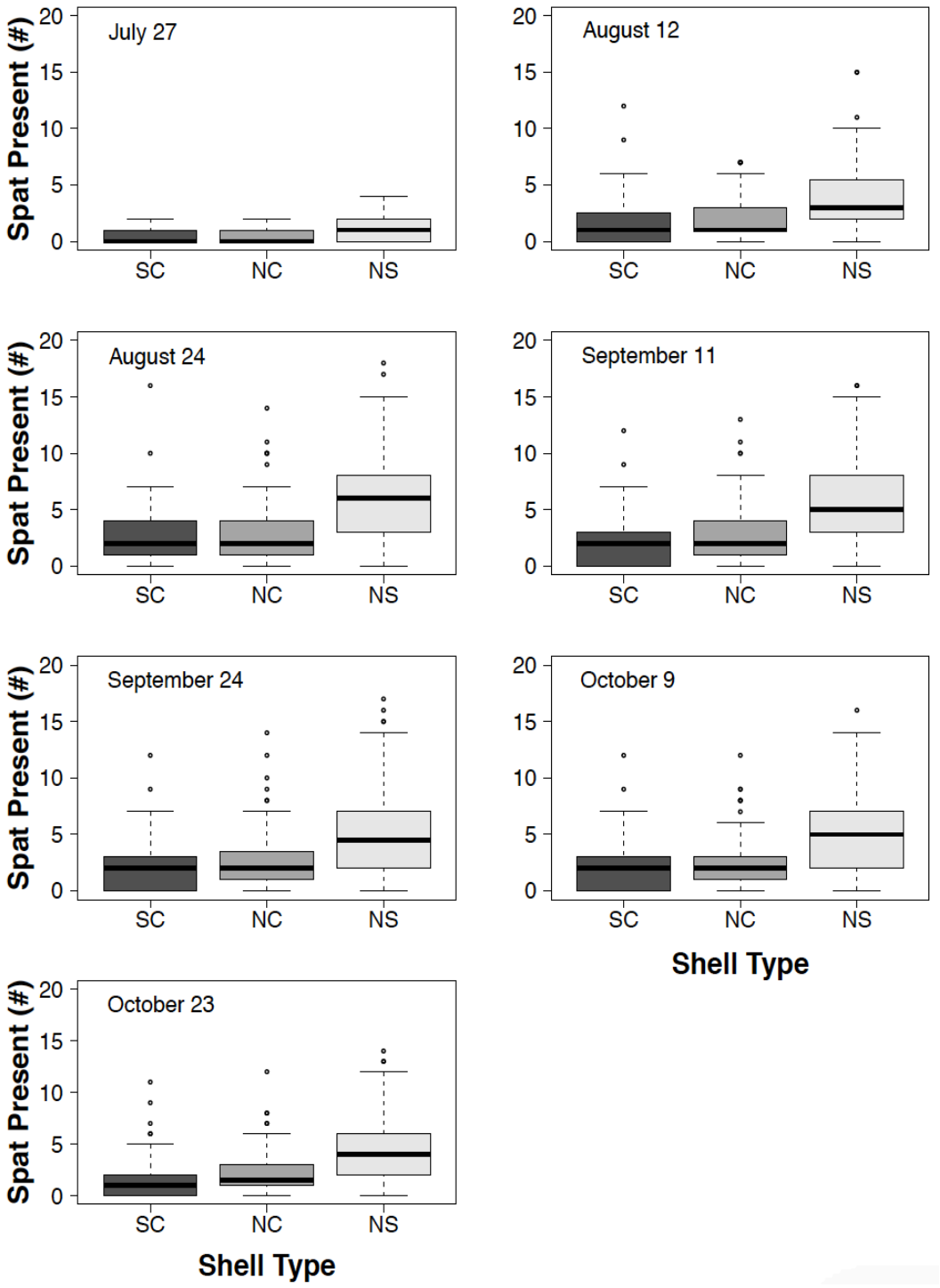

Figure 2.8. Settlement Distributions The distribution of larval settlement greatly varied between treatments and between collection dates. Spat counts were recorded on the designated dates. Each boxplot includes data from 80 experimental units. Shaded boxes represent the 25% to 75% distribution. Lower and upper bounds represent the full range of data, respectively. Dark bands represent the sample median. Dots represent sample outliers. SC = special concrete, NC = normal concrete.

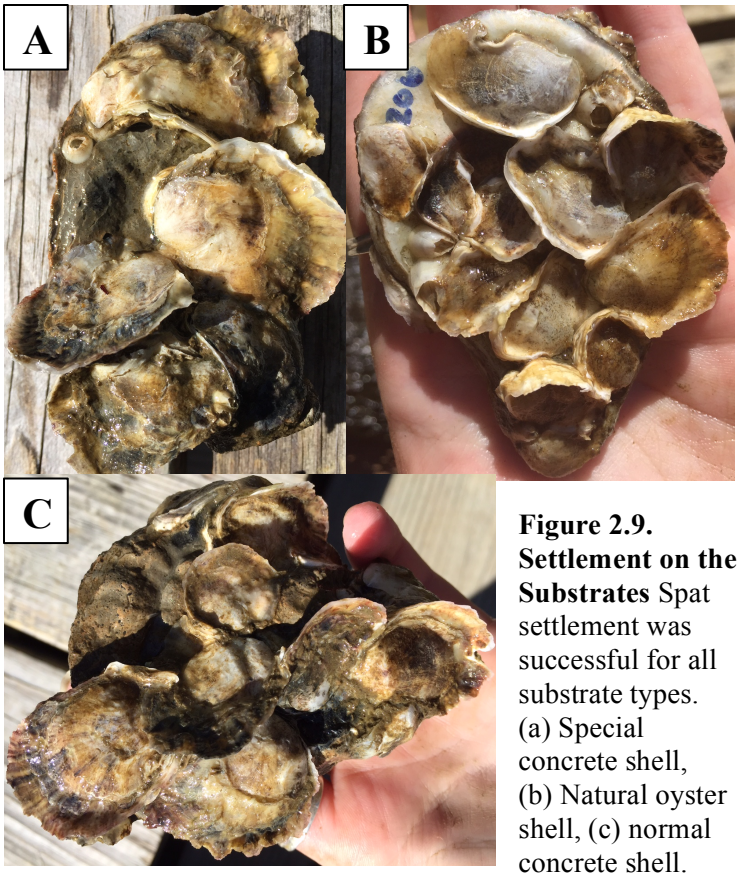

Figure 2.9. Settlement on the Substrates Spat settlement was successful for all substrate types. (a) Special concrete shell, (b) Natural oyster shell, (c) normal concrete shell.

Settling Preference and Abundance

Overall, spat settled on the special concrete (n = 149) and normal concrete shell treatments (n = 174) less frequently than on the natural shell treatment (n = 365; Chi-square at end of study; $p < 0.001$). This suggests that the oyster larvae have a clear preference for natural shells as a settling substrate compared to the concrete treatments.

Due to similar skews within treatments (Figure 2.8), spat counts for each shell treatment were pooled for each collection date (Shapiro-Wilk; all $p < 0.001$). Each collection date was analyzed separately from each other to observe the pattern of spat presence on each substrate type. On the first collection date (July 27, 2016), the special concrete had statistically significantly fewer spat (n = 36) than the natural shell treatment

(n = 80; Kruskal-Wallis; $p < 0.05$; Figure 2.10). There was no statistical difference in spat count between the natural shell and normal concrete shell treatment (n = 40) at that time (Kruskal-Wallis; $p = 0.069$; Figure 2.10). From August 12 to October 23 (remaining collection dates), special concrete and normal concrete shell treatments had significantly fewer spat than natural shell (Kruskal-Wallis; $p < 0.001$). Total spat counts were not statistically different between special concrete and normal concrete shell treatments at any collection time (Kruskal-Wallis; all $p > 0.6$; Figure 2.10).

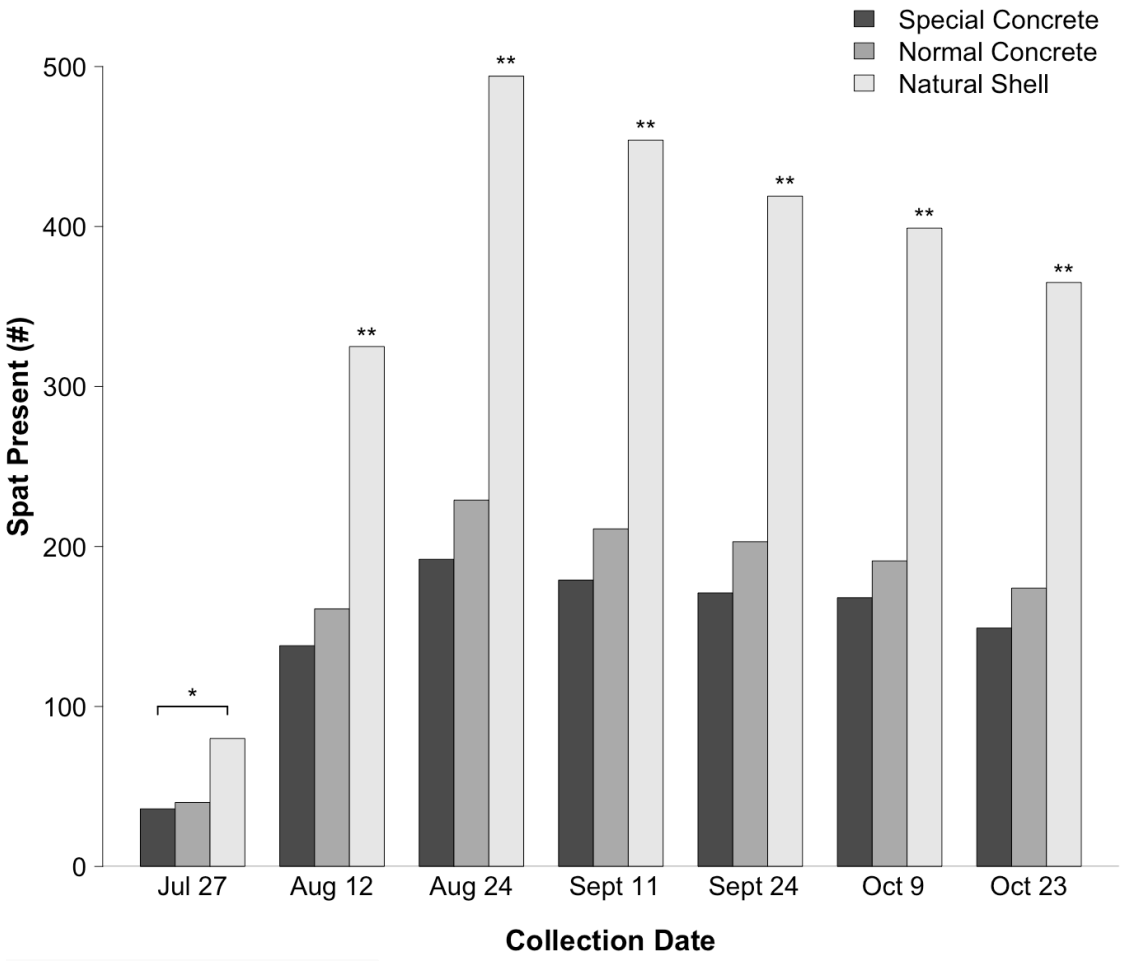

Figure 2.10. Spat Presence Over Time Number of spat present at each collection period on 80 units per shell type. Spat counts were collected in Pitman's Cove in Kilmarnock, VA. Statistical analyses were completed using a Kruskal-Wallis test. Comparisons were made between shell treatments at each individual collection period. * = $p < 0.05$ ** = $p < 0.001$.

Change in Abundances Over Time

There was an initial spike in percent spat settlement for all treatments between the first and second collection dates (July 27 to August 12). The special concrete treatment had a 283% increase, normal concrete had a 302% increase, and natural shell had a 306% increase in spat settlement (Figure 2.11; Table 2.2). Between the second and third collection dates (August 12 to August 24), there was another increase in percent spat for all treatments, although not as pronounced (Figure 2.11; Table 2.2). Special concrete saw a 39% increase, normal concrete saw a 42% increase, and natural shell saw a 52% increase in spat settlement (Figure 2.11; Table 2.2).

For all subsequent collection dates (Aug 24-Sept 11, Sept 11-Sept 24, Sept 24-Oct 9, Oct 9-Oct 23) there was a percent decrease in spat on each treatment (Figure 2.11; Table 2.2). The special concrete shells had an average decline in total spat of 6.23% (SD = 4.24, total loss of 43; Table 2.2) per collection period. The normal concrete treatment had an average decline in total spat of 6.63% (SD = 2.26, total loss of 55; Figure 2.11; Table 2.2) per collection period (Figure 2.11; Table 2.2). Finally, the natural shell treatment had an average decline in total spat of 7.28% (SD = 1.68, total loss of 129; Figure 2.11; Table 2.2) each collection period. However, there was no statistical difference between the percent declines of spat between the three treatment types (ANOVA; $p = 0.88$) for the final four collection periods.

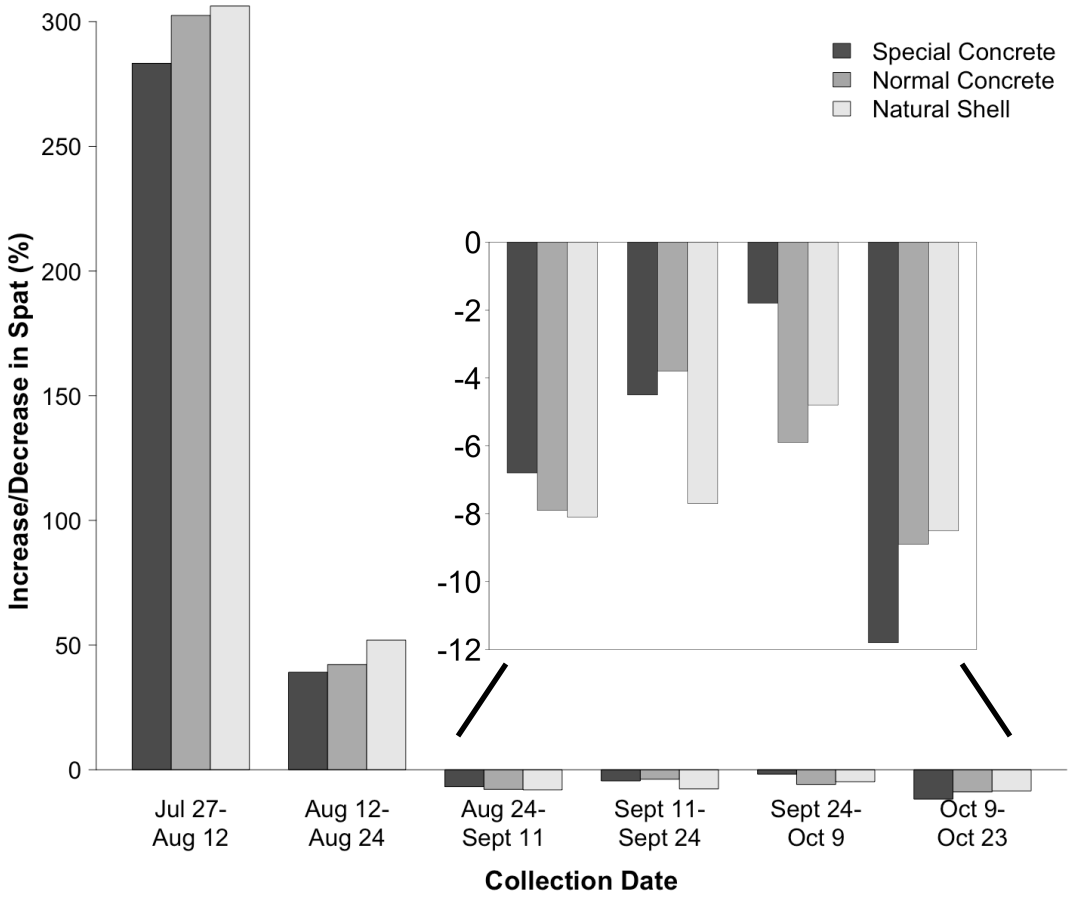

Figure 2.11. Change in Presence Over Time Percent increase or decrease in total spat on each treatment type between collection periods. Each treatment contains 80 experimental units. Initial settlement on SC = 36, NC = 40, NS = 80.

Table 2.2. Percent Increase/Decrease Percent increase (+) or decrease (-) in total spat on each treatment type between collection periods. Initial settlement on SC = 36, NC = 40, NS = 80.

	Collection Period					
Substrate	**July 27-Aug 12**	**Aug 12-Aug 24**	**Aug 24-Sept 11**	**Sept 11-Sept 24**	**Sept 24-Oct 9**	**Oct 9-Oct 24**
Special Concrete	+283.3	+39.1	-6.8	-4.5	-1.8	-11.8
Normal Concrete	+302.5	+42.2	-7.9	-3.8	-5.9	-8.9
Natural Shell	+306.3	+52.0	-8.1	-7.7	-4.8	-8.5

Discussion

The goal of this study was to determine the larval recruitment success of an artificial substrate that had augmented levels of calcium to better mimic natural oyster shells for restoration purposes. To gauge success, we analyzed the settling preference of spat, the settling abundance of spat, and the change in abundance of spat. We predicted that spat would prefer to settle on the natural shell treatment and there would be more settlement on the special concrete compared to the normal concrete.

Spat did prefer to settle on the natural shells, as predicted, with more than double the larval settlement found on that substrate compared to the special and normal concrete treatments. Spat settlement was similar between the special and normal concrete shell treatments but much higher on the natural shell treatment. The results of this study are similar to other studies that showed greater spat recruitment to natural shell relative to artificial substrates (Tamburri et al. 2008**,** Dunn et al. 2014, Theuerkauf et al. 2015). Throughout the study period, the percent decline in spat count on all treatments was similar. However, looking at raw numbers, the natural shell treatment lost more than twice as many spat over the course of the study compared to the other concrete treatments.

Settling Preference and Abundance

Spat preferred to settle on the natural shell treatment rather than the concrete treatments. Free-swimming larvae across taxa are able to actively choose where they settle (Crisp 1967, Roberts et al. 1991, Woodin 1991, Tamburri et al. 1992, Faimali et al. 2004, Tamburri et al. 2008, Wilkie et al. 2013). In this case, we suspect that oyster spat

preferred to settle on the natural shell treatment for several potential reasons. First, biofilm communities that form on substrates vary due to the chemical makeup of the substrates (Faimali et al. 2004). Oysters potentially prefer the chemical biofilm that forms on natural oyster shells because it can be associated with a more ideal habitat (Crisp 1967, Tamburri et al. 2008). This could also, in part, be due to the specific trace metals found in that substrate contributing to the composition of the biofilm compared to the concrete treatments. Secondly, the natural occurrence of calcium, rather than the artificial addition of calcium, might induce spat to settle on natural oyster shells more frequently relative to the special concrete treatment (Soniat et al. 1991). Thirdly, research suggests that oysters prefer interstitial space that provides an ideal habitat for spat settlement, protection from predation, and shelter from abiotic stresses (Bartol et al. 1999, Gutierrez et al. 2003, Grabowski 2004, Nestlerode et al. 2007); the interstitial space provided by natural shells could have been more conducive to spat settlement and subsequent survival. The concrete shell units were more bulky, since they were casts of full shells, and did not allow for as much interstitial space between units in the mesh bags. The natural shells used this study were only half shells, therefore allowing for more interstitial space for spat settlement when in the mesh bags. Finally, the nacre, the smooth inner surface of oyster shells, has a different texture and composition than the rest of the oyster shell. Most of the settled spat on the natural oyster shells were on this surface rather on the rough outer portion of the shells. Therefore, the smooth nacre could also be an attractive feature of the natural shells compared to the more rough surface of the concrete treatments, therefore attracting more settlement.

Change in Abundances Over Time

There was an initial increase in spat settlement on all treatments. However, there was a percent decline in total spat for all treatments. Even though the percent decline in spat did not differ between treatments, the raw numbers tell a different story. Although the natural shell ended the study with more settled spat, they still lost more than twice as many spat compared to the two concrete treatments.

One explanation for the greater initial spat settlement, but the eventual greater decline of total spat, on the natural shell treatments could be explained by the theory of Ideal Free Distribution (Fretwell and Lucas 1970, Fretwell 1972). This theory states that organisms will distribute themselves across several patches of habitat based on the quality of the site so that the resulting fitness at the sites is equal (Fretwell and Lucas 1970, Fretwell 1972). Patches of habitat with more resources will have more organism settlement and habitats with fewer resources will have fewer organism settlement. However, overall fitness is reduced with high settlement because there is more competition for available resources. Therefore, even though an "ideal" habitat has the most resources, higher population densities in that habitat will effectively reduce the overall fitness of the organisms present.

For oysters, natural oyster shells are considered to be the most ideal habitat. Two important recourses for newly settled spat are space to grow and access to food. As more spat settled on the natural shell treatment, less space was available for the spat to grow and there was less access to food, inducing competition (Figure 2.12). This competition may have reduced the overall fitness of the spat that settled on the natural shell treatments, which, in turn, could have resulted in the steady decline of spat over the study

period (Figure 2.11). In contrast, the normal and special concrete shell treatments were less "ideal" and, therefore, had less total settlement, which made the subsequent decline in total spat more pronounced resulting in the difference in percent decline being non-

Figure 2.12. Variable Settlement The space available to spat on the different substrate types was variable. More space allowed for the oyster to have a stronger hold on the substrate (top) compared to oysters that had to compete for space and grow outward (bottom). Pictures were taken on August 24, 2016.

significant. The lower settlement led to more available space for shell growth and more access to food (Figure 2.12). As time progressed, the spat that initially settled on the normal and special concrete shell treatments were able to survive longer resulting in more consistent spat counts throughout the study period (Figure 2.10).

The theory of ideal free distribution has been supported for another Chesapeake Bay species, the marine clam, *Macoma balthica,* (Meer et al. 2013). Meer et al. (2013) estimated the lifetime egg production, as a proxy for overall fitness, of *M. balthica* over 30 years at a gradient of ideal habitats. They found that free-swimming larvae that settled in a more ideal habitat had a higher fitness but lower long-term survival. Therefore, the larvae had a trade-off between long-term survival in a harsh environment with more

opportunities but less successful reproduction, and short-term survival in an ideal environment with fewer opportunities but extremely successful reproduction. The clam findings mirror what was seen in the current study with larvae choosing an ideal habitat, natural shell, despite having a lower chance of long-term survival potentially due to competition for space to grow. We currently do not have the long-term settlement and survival data for this study since data were only collected during one spawning and recruitment period. Despite this, the short-term patterns show that the greater initial frequency of spat settlement on the natural shell will cause greater declines due to limited resources for further growth.

Another possible reason for the steady decline in total spat on the natural shell could be predation. As time progressed, there was a higher prevalence of shells that were open with no live tissue, broken with no live tissue, or only consisted of one side of the shell indicating mechanical predation had occurred (Figure 2.13). During the study period, there were Blue crabs (*Callinectes sapidus*) present, which are known to prey on young oysters, at the field site (McDermott 1960, Krantz and Chamberlin 1978, Bisker and Castagna 1987). With higher juvenile densities on the natural shell treatment, Blue crabs may have been more attracted to the natural shells, as a meal would have been easier to acquire because the growing oysters were sticking out of the holes in the mesh bag. Conversely, less dense juvenile oysters on the concrete treatments would have been less attractive because Blue crabs would have had to put more energy in acquiring a meal. In addition, because there was potentially less interstitial space available for the spat on the concrete treatments, those that settled were not as protected from predators because they were forced to be in the open. Therefore, it would have been easier for the Blue

crabs to feed on juveniles that settled on the concrete treatments compared to the more protected juveniles on the natural shells.

Figure 2.13. Predation on Natural Shells Predation was evident due to broken shells and the absence of living tissue.

Future Studies

Several potential studies stem from the results of this study. First, measuring the growth of the shells (mm) of spat overtime could reveal differences in the nutrients available on each shell treatment. Spat are attracted to substrates that contain calcium (Soniat et al. 1991, Soniat and Burton 2005), however, the long-term effects of a substrate containing supplemented calcium on growth, survivorship, and reproduction are still unknown. The augmentation of calcium in the special concrete shells might provide the settled spat with the calcium needed to build their shells. Due to ocean acidification, there is less calcium carbonate available in the water column (Orr et al. 2005) making it harder for bivalves to make strong outer shells (Kurihara and Ishimatsu 2007). Providing access to supplementary calcium in such close proximity, the spat on the special concrete shell treatment could show healthier growth over time compared to the spat on the natural shell and normal concrete shell treatments.

In conjunction with growth, monitoring the survival rate of settled spat on the varying shell treatments could reveal if the health of the spat is related to the settling substrate. In order to determine the long-term success of the substrates used in this study, the survival of the settled oysters would need to be monitored until adulthood (~3 years). With more time there could be a more pronounced decline of spat on the natural shell compared to the concrete treatments. There are a few studies looking at the effect of substrate type on long-term spat survival (Manoj Nair and Appukuttan 2003, Nestlerode et al. 2007, Drexler et al. 2014, Theuerkauf et al. 2015), but the effect of the chemical makeup of the substrate and how it relates to oyster survival over time is largely unexplored. Subtle differences in the chemical makeup of settling substrates could have drastic and lasting effects on reproductive success, shell durability, and life expectancy. By exploring the long-term growth and survival of spat that settled on substrates with varying chemical makeups, we can better understand oyster restoration and, in turn, make it more successful in the future.

Finally, monitoring localized water quality and algal growth in the presence of the special concrete treatment could reveal benefits or risks in using this as an artificial substrate for oyster restoration. The addition of limestone to rivers and streams has been shown to maintain pH levels and, potentially, mitigate acidification (Menendez et al. 1996, Clayton et al. 1997, Hudy et al. 2000). It is unknown if the artificial addition of calcium into marine systems has the same affect. Therefore, it is critical to monitor the breakdown of the special concrete shells over time. As the special concrete shells break down, they might release calcium into the surrounding environment, allowing for the localized mitigation of ocean acidification.

Conclusions

This study clearly demonstrates that oyster spat prefer to settle on natural shell to artificial substrates. Other studies have shown that concrete is successful for oyster restoration (Theuerkauf et al. 2015, Dunn et al. 2014, Drexler et al. 2014). However, anecdotal evidence suggests that oysters do not have long-term success on normal concrete artificial substrates. While we expected natural shell to be the preferred substrate, the relative larval recruitment success of the two concrete substrates is promising. Although the total spat count was lower, the concrete shell treatments were still successful in recruiting a similarly substantial number of spat throughout the study period. Therefore, initial data suggest that the addition of calcium to artificial substrates does not show adverse effects in oyster larval recruitment. Long-term studies need to be conducted to ensure the same anecdotal effects of normal concrete on oysters are not seen on the special concrete artificial substrate.

When taking into account the short supply and elevated cost of acquiring natural shells for restoration, concrete casts, with or without calcium augmentation, are more economically practical because of the more consistent spat counts throughout the entirely of the study period compared to the steady decline of spat on the natural shell treatment.

III. Effect of Artificial Substrates on Water Quality and Algal Growth

Abstract

Oyster restoration in the Chesapeake Bay is needed to combat population declines caused by over harvesting, disease, ocean acidification, and poor water quality. Oyster restoration efforts in the past have used natural oyster shells to quickly increase local populations but doing so is expensive and unsustainable. There has been a shift towards using artificial substrates instead. As with any restoration effort, it must be ensured that there is no subsequent harm to the environment. However, most studies that examine artificial substrates for restoration focus solely on efficacy. Few studies looked at the effects of artificial substrates on localized water quality or on trophic interactions that are present in conjunction with oyster reefs. This study examined the effect of artificial substrates (special concrete, normal concrete, and natural oyster shells), all of which have shown success in oyster larval recruitment, on localized water quality and algal growth. Each substrate was placed in aquaria with three replicates each. Water quality in the aquaria in the presence of these substrates was measured (pH, conductivity, alkalinity, and calcium) for a 12-week period. Water samples were taken at the start, mid, and end of the 12 week period in which five algal strains were grown. Fluorescence and Fv/Fm (difference between minimum and maximum fluorescence) were measured as proxies for growth and cell stress respectively. There was an effect of substrate on pH with the special concrete causing significantly lower values (8.27) than the normal concrete (8.32; $p = 0.0015$) and natural shell treatments (8.45; $p < 0.001$). Both concrete treatments had an effect on alkalinity values with the special concrete (133.3 ppm) and normal concrete treatments (130.9 ppm) causing significantly lower values than the natural shell treatment

(196.3; $p < 0.001$). However, the pH and alkalinity values were not biologically significant because they were within the range of normal seawater. The artificial substrate with augmented calcium supported lower growth of the algal strains but this was only slightly lower than the other concrete substrate. Initially, it appears that both concrete treatments do not cause immediate harm to the surrounding environment. Long-term studies should be conducted to confirm these results for long-term artificial substrate use in the Chesapeake Bay.

Introduction

The Need for Oyster Restoration

In the Chesapeake Bay, native oyster populations are below 1% of their historical quantities (Beck et al. 2011, Dunn et al. 2014; Figure 3.1). The main causes for this drastic decline in oyster population size are overharvesting, disease, ocean acidification, and poor water quality (Cook et al. 1998, Kemp et al 2005, Woods et al. 2005, Mann et al. 2009, Carnegie and Burreson 2011, Dunn et al. 2014).

*Over Harvesting***.** The most substantial impact to oyster populations in the past, and even presently, is over harvesting. The Chesapeake Bay was once the most productive oyster-harvesting site in the world (Woods et al. 2005). In the late 19th century to the early 20th century, Virginia harvested an average of 6 million bushels of oysters each year (Woods et al. 2005). Woods et al. (2005) studied the result of overharvesting on 20 reefs in the James River, which remains one of the most productive oyster producing rivers in the Chesapeake Bay. They found that an average of 18,000 cubic meters of oysters and

shells were removed from each of the 20 reef systems because of overharvesting between 1870 and 1940. These oysters were never replenished once they were taken which caused a decline in the total number of oyster reefs in the Chesapeake Bay (Woods et al. 2005; Figure 3.1).

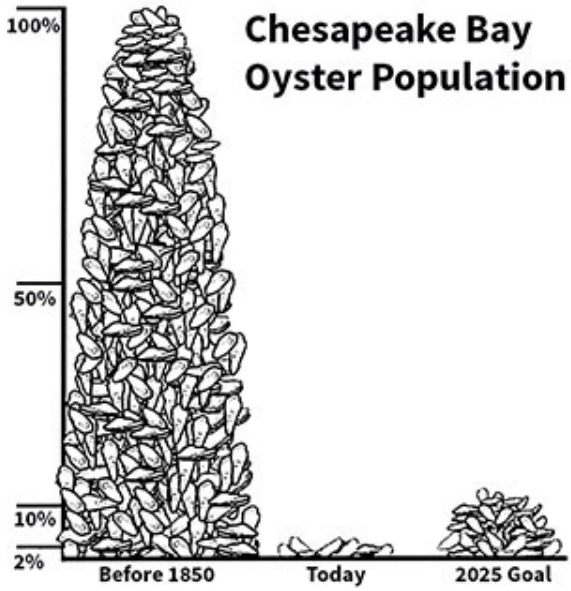

Figure 3.1. Oyster Population Decline Oyster populations in the Chesapeake Bay have declined by 99% since the 1800's due to overharvesting. Figure from Ocean Cove Seafood.

Disease. In the 1950's, there was a breakout of two parasitic diseases, *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo), that caused 90% mortality in oyster reefs in the Chesapeake Bay (Rothschild et al. 1994, Kemp et al. 2005, Carnegie and Burreson 2011). Even though many of the current oyster populations have developed a natural resistance to these diseases due to heavy infection pressures in the past, those who have not are still susceptible to infection (Carnegie and Burreson 2011).

Ocean Acidification (OA). Climate change has led to serious negative consequences in marine ecosystems. The ocean has become a carbon sink due to excess carbon dioxide in the atmosphere from fossil fuel emissions (Orr et al. 2005). The surplus of dissolved carbon dioxide combines with water in the ocean to made carbonic acid according to the following equation:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \qquad \text{Equation 1}$$

The carbonic acid formed in this reaction then creates bicarbonate ions and hydrogen ions (Figure 3.2). An increase in concentration of free hydrogen ions leads to decreased pH levels (more acidic).

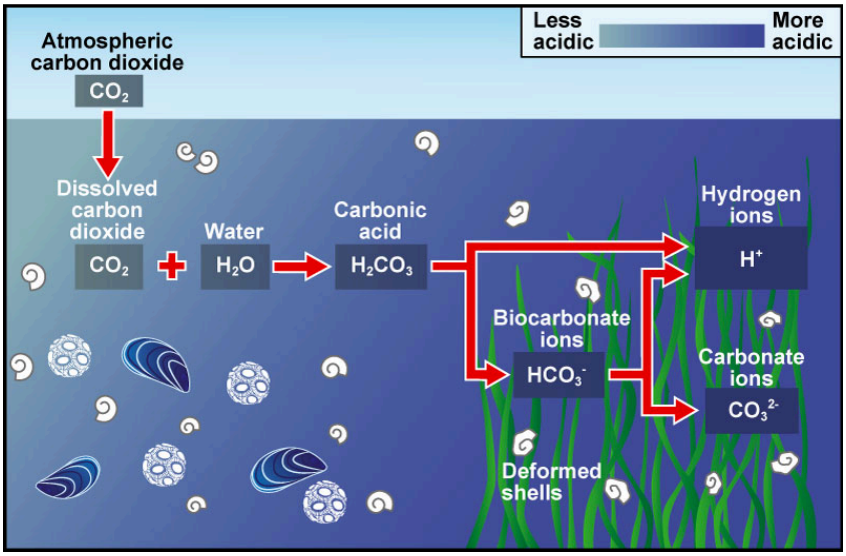

Figure 3.2. Ocean Acidification Ocean acidification is caused by dissolved CO2 reacting with seawater resulting in an excess of free hydrogen ions. Figure from the UK Ocean Acidification Research Programme.

The severity of trophic response to OA and ocean warming is difficult to estimate but is expected to be far-reaching (Kroeker et al. 2013). Effects could range from positive outcomes like increased growth rates of macro algae due to increased available carbon dioxide (Koch et al. 2012), to negative outcomes like reduced calcification rates of

bivalves due to the reduced concentrations of available calcium carbonate (Talmage and Gobler 2010). Ocean acidification research has greatly expanded in the past decade (Kroeker et al. 2013) and researchers are trying to understand OA's consequences on ecosystems, address management concerns, and make restoration goals to mitigate the effects of OA.

In the Chesapeake Bay, the majority of research on the effects of OA has been focused on oysters because of their economic importance (Bahr and Lanier 1981). Talmage and Gobler (2009) found that *Crassostrea virginica* larvae have reduced larval growth when exposed to lower pH (7.50) and show reduced subsequent survival. The pH values in their study were chosen based on a projection of dissolved CO_2 levels in the ocean (increased CO_2 leads to decreased pH) in the year 2250 (Talmage and Gobler 2009). Other species of oysters have shown similar results when under the same conditions (Talmage and Gobler 2009). In addition, increased concentrations of carbon dioxide likely leads to a decreased concentration of available carbonate ions (Orr et al. 2005). CO_2 and H_2O combine to create H_2CO_3 (carbonic acid) which then breaks into bicarbonate and hydrogen ions (Figure 3.2). However, the percent increase in hydrogen ions in this step is much larger than the percent increase in bicarbonate ions (Orr et al. 2005). This imbalance requires available carbonate ions to bind with the free hydrogen's to maintain chemical equilibrium. Therefore, the concentration of carbonate ions is greatly reduced with an increase in dissolved CO_2. Calcifying organisms, such as oysters and other bivalves, need carbonate, in the form of calcium carbonate, to make their shells. If there is a low concentration of carbonate ions, then oysters cannot efficiently make the shells needed to survive. Kurihara and Ishimatsu (2007) found that 70% of oyster larvae

kept in a control environment with a normal carbonate concentration of 161.4 µmol/kg were fully covered in a calcium carbonate shell 48 hours after fertilization (Figure 3.3). However, for oyster larvae kept in an environment with the carbonate concentration reduced to 36.4 µmol/kg (projection for 2300), only 30% had fully developed calcium carbonate shells 48 hours after fertilization (Kurihara and Ishimatsu 2007; Figure 3.3).

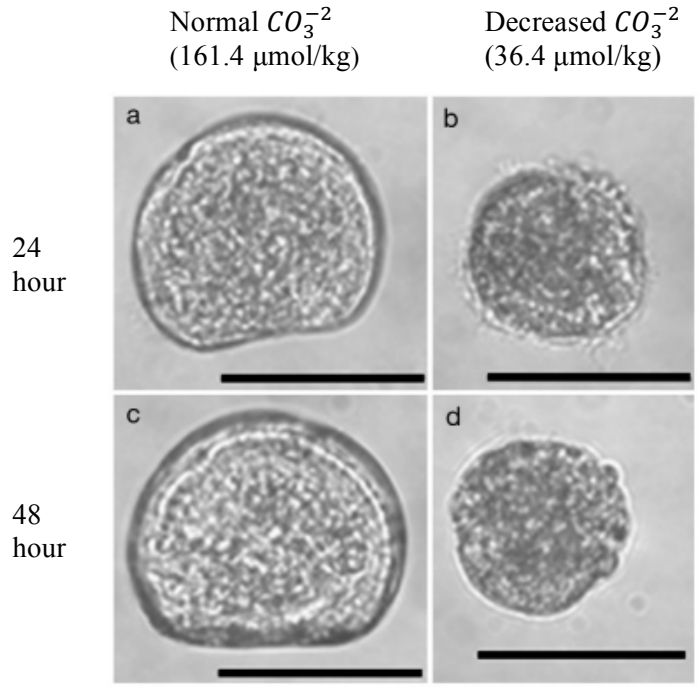

Figure 3.3. Effect of Reduced Carbonate Oyster larvae show reduced shell growth when exposed to decreased concentrations of available carbonate. (a) 24h larvae under normal conditions, (b) 24h larvae under decreased CO_3^{-2}, (c) 48h larvae under normal conditions, (d) 48h larvae under decreased CO_3^{-2}. Scale bar = 50mm. Adapted from Kurihara and Ishimatsu 2007.

The dangerous combination of low pH and low concentrations of carbonate due to OA has current and future implications to oyster populations. As global warming increases, OA is projected to intensify, and as a result, oyster populations will decrease. This calls for restoration that takes into account the effects of OA in order to maintain oyster populations in the Chesapeake Bay.

Water Pollution. Human expansion in areas near ocean shorelines greatly increased between 1870 and 1950 (Sindermann 1995). This influx of human activity led to an influx of sediment, sewage, toxic chemicals, and agricultural runoff into marine ecosystems (Sindermann 1995, Gottlieb and Schweighofer 1996).

Pollution of the Chesapeake Bay is of great concern because the Bay was once the most productive oyster-harvesting site in the world (Woods et al. 2005). However, only 1% of their historic quantity remains due, in part, to water pollution (Dunn et al. 2014). Oysters are filter feeders, syphoning the surrounding water containing food and any chemicals (Bahr and Lanier 1981). Evidence suggests that pesticide and herbicide runoff still affects oysters in the Chesapeake Bay despite regulations that have reduced the level of pollution (Lehotay et al. 1998, Kannan et a. 2002). Therefore, restoration efforts are required to return oyster populations to sizes they once were.

Typical Goals of Oyster Restoration

Past oyster restoration efforts have only been concerned with quickly increasing populations to exploit the benefits that oyster provide, including improving water quality (Drexler et al. 2013, Kellogg et al. 2013), creating living shorelines (Theuerkauf et al. 2015), and supporting high biodiversity (Peterson et al. 2003). In addition, restoration efforts have focused on using natural oyster shells as the settling substrate (NERI), but as populations continue to decline, oyster shells become more rare and more expensive (Mann and Powell 2007). Therefore, restoration work increasingly shifts towards using artificial substrates, specifically concrete for restoration purposes. Most research using concrete as an artificial substrate has only focused on the efficacy of concrete as a

supporter of larval settlement (Tamburri et al. 2008, Whitman and Reidenbach 2012, Drexler et al. 2014, Dunn et al. 2014, Theuerkauf et al. 2015). Further research needs to be conducted to ensure artificial substrates are not causing harm to the immediate water quality or to the trophic interactions that are present in conjunction with oyster reefs.

Possible Effects to Water Quality

Natural oyster reefs have been suggested to improve localized water quality through their natural filter feeding processes (Coen et al. 2007, Grabowski and Peterson 2007). But because populations are so low, artificial substrates, specifically concrete, are now being used as the basis for many reef systems. However, introducing an exotic physical habitat made from concrete might negatively affect local water quality and negate the positive effects that oysters provide.

Other artificial substrates have been examined for their effects on water quality in aquatic systems outside of oysters (Azim et al. 2002, Azim et al. 2003, Moss and Moss 2004, Arnold et al. 2006, Scheitzer et al. 2013). Azim et al. (2002) were concerned with how artificial substrates (bamboo, kanchi, and hizol tree branches) affected pH and alkalinity for the growth of periphyton. They found that in the presence of these exotic substrates, the pH and alkalinity were not significantly affected (Azim et al. 2002). Moss and Moss (2004) also saw that water quality (water temperature, pH, and dissolved oxygen) was unaffected by the presence of artificial substrates (AquaMats - polymer filters) being used to improve white shrimp aquaculture. Literature on negative effects to water quality in the presence of concrete substrates is limited potentially due to research

on effects of concrete on water quality not being performed or researchers not publishing negative/adverse or null results.

Several studies show that oyster reefs create habitats that allow for a diverse array of vertebrate and invertebrate organisms that directly benefit from the oysters complex manufacturing of reefs (Behr and Lanier 1981, Peterson et al. 2003, Grabowski and Peterson 2007). For example, various species of fish, like gobies and seatrout, and invertebrates, like polychaetes and crustaceans, often use oyster reefs as foraging grounds and refuge from predators or during periods of high-energy wave action (Boudreaux et al. 2006, Coen et al. 2007, Grabowski and Peterson 2007, Soniat et al. 2007). Concrete as an artificial substrate for oyster growth has potentially negative effects on water quality due to the trace metals found in the concrete formula. If the base of an oyster reef is constructed from concrete substrates that cause diminished localized water quality, the ability of this reef to support these high levels of biodiversity greatly reduces. In addition to the reduced level of biodiversity around the reef system, poor water quality induced by the artificial substrates could lead to reduced oyster populations, adding to the problem already facing oyster populations. Again, little research has been conducted on how artificial substrates effect oyster reefs so the realized consequences may be more, or less, severe than described; the effects are still largely unknown and are initially examined in this study.

Trophic Interactions on Oyster Reefs

There are two main types of trophic interactions: top-down controlled trophic cascades and bottom-up controlled trophic cascades. Top-down trophic cascades

function with the assumption that the abundance of lower food web elements are controlled by upper food web predators (Paine 1980, Pace et al. 1999). Therefore, with a reduction in upper level predators, it is assumed that there will be an increase in the abundance of lower food web elements because there are less predatory pressures (Pace et al. 1999). Bottom-up trophic cascades function on the assumption that lower food web elements control the abundance of upper food web predators (Pace et al. 1999). With a reduction in lower food web elements, the upper food web predators would face limited food availability (Pace et al. 1999). In natural ecosystems, it is difficult to separate these hypotheses because they rarely function discretely (Menge 1992, Ware and Thomson 2005, Burkepile and Hay 2006). Therefore, it is presumed that both of these trophic cascade patterns are present on oyster reefs.

Predators, like crabs and oyster drillers, prey on oysters and control the abundance of oysters on reef systems in a top-down manner (Muthiah et al. 1987; Figure 3.4). Algae that develop on oyster shells and in the surrounding water is consumed by primary consumers, like copepods, which are then consumed by secondary and tertiary consumers, like crabs and large fish (CBP – Food Web; Figure 3.5). This is a bottom-up cascade because the abundance of algae impacts the upper food web constituents. Any direct impact on the top predators or algae growth could have effects on both the top-down and bottom-up trophic cascades that are associated with oyster reefs.

Figure 3.4. Oyster Prey Typical predators of oysters are blue crabs and oyster drillers. Figures from the South Carolina Department of Natural Resources and the Jacksonville Shell Club.

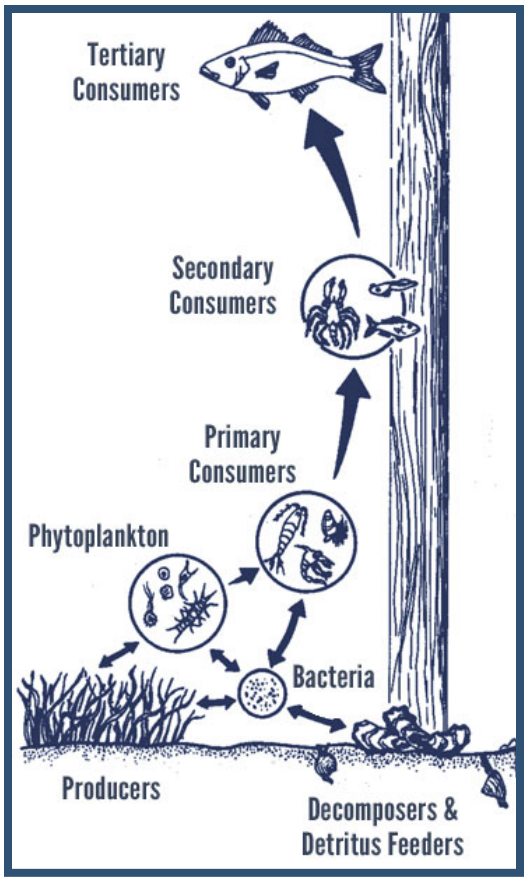

Figure 3.5. Oyster Food Chain Oysters are involved in a bottom-up trophic cascade that starts with algae growth on the shells and ends with tertiary consumers like large fish. Adapted from the Chesapeake Bay Foundation – Food Web Guide.

In this study, I examined the growth of several algal species in the presence of artificial substrates to determine if the substrates were releasing any elements that would prevent propagation, potentially leading to disruption in the bottom-up trophic cascade. There are examples of how bottom-up trophic cascades are affected when the lower food web elements are disturbed (Ware and Thomson 2005). Ware and Thomas (2005) used satellite images to determine chlorophyll-a (chl-a) concentrations (proxy for algal growth) along the west coast of the U.S. and correlated this with fish yield in the same areas. They found that with lower concentrations of chl-a, there was a statistically significantly lower mean fish yield. This suggests that a bottom-up trophic cascade controls the upper food web predators and that any changes in the abundance of the lower food web elements can greatly affect the abundance of the larger predators (Ware and Thomas 2005). If this relationship is present on reef systems, specifically if the reduction in algal growth controls the abundance of upper food web constituents, then researchers should ensure that any artificial substrate being introduced are not causing harm to this sensitive bottom-up trophic cascade.

Effect of Varying Concrete Formulations on Water Quality

The use of varying concrete chemical formulations has not been addressed in oyster restoration research. Previously, I examined the efficacy of an artificial substrate with augmented levels of calcium, to better mimic natural oyster shells, on larval recruitment. I found that there were no initial adverse effects on larval recruitment on concrete with augmented calcium. However, before this substrate can be determined a possible substrate for oyster restoration, the effect of this substrate on localized water

quality needs to be examined. If this substrate negatively affects local water quality, then it should not be considered as a viable substrate for restoration. Typical parameters to assess water quality are pH, conductivity (μS/cm), alkalinity (ppm CaCO3), and calcium (ppm; WHO 1996).

Effect of Varying Concrete Formulations on Algae Growth

As described above, algal growth in the presence of artificial substrates has not yet been explored. If the substrates are releasing a harmful chemical/element that prevents the growth of algae, there could be drastic bottom-up tropic cascade effects near oyster reef systems. In order to determine the effect of these artificial substrates, several algae strains known to be in the Chesapeake Bay were grown in media that contained each of the substrates. Two measures of algal success were evaluated, the fluorescence as a proxy for growth and the photosystem efficiency (Fv/Fm) as a proxy for cell stress.

Fluorescence is a measured by shining a beam of light onto a sample and measuring how much of that light is emitted back. Fluorescence is typically used as a proxy for cell growth with higher fluorescence signifying greater growth. (ie. more cells emitting light back; Wood et al. 2005). Algae with high levels of fluorescence are considered to be growing healthy. If there is little fluorescence being measured, this suggests that the alga is not growing as efficiently as it could, potentially due to the environment it is in (Mayer et al. 1997). Using fluorescence measures could determine if the artificial substrates used in this study were off-putting anything that could prevent algal growth.

In addition to fluorescence, Fv/Fm was also measured. Fv/Fm is a measure of the efficiency of photosystem II and is a proxy for cell stress. It is calculated by the following equation:

$$\frac{Fv}{Fm} = \frac{Fm-Fo}{Fm} \qquad \text{Equation 2}$$

where Fm is maximum fluorescence, Fo is minimum fluorescence, and Fv is the difference between Fm and Fo. It is measured, first, by determining the minimum fluorescence when all cells are dark-adapted and not allowed to begin photosynthesis. Then, saturating light is shown onto the sample and maximum fluorescence is measured under the most ideal conditions. Finally, using these two values, the Fv/Fm value is calculated by subtracting the minimum fluorescence from the maximum fluorescence and dividing by the maximum fluorescence. The final value represents the maximum potential efficiency of photosystem II in that particular sample. Fv/Fm is a proxy for stress in algae (Liu and Huang 2000, Parkhill et al. 2001, Stepien and Klobus 2006). Lower Fv/Fm measurements signify that the algae are unable to absorb light efficiently because they are under stress (Liu and Huang 2000, Parkhill et al. 2001). Conversely, if the Fv/Fm measurements are high, then the algae are able to absorb light and use it efficiently, signifying they are not under any significant stress.

The goal of this study was to examine the effects of three substrates on water quality and algal growth: (1) special concrete with augmented levels of calcium, (2) normal concrete, and (3) natural oyster shells. Specifically, I tested the hypothesis that all three treatments showed no adverse effects on water quality measured by pH, conductivity, alkalinity, and calcium. In addition, I predicted that the growth and stress levels of algal species grown in water that once contained these treatments will not be

affected. The measures of water quality and algal growth combine to tell a more complete story about the quality of the substrates being used for restoration, rather than the efficacy of them.

Materials and Methods

Manufacturing of Shells

Concrete substrates (calcium augmented and normal) were manufactured in the same manner as the previous chapter. Fifteen special concrete shells and fifteen normal concrete shells were manufactured. Along with this, 15 natural oyster shells were acquired.

Preparation

Shells and Aquaria. Each experimental unit (shell) was given a unique identifying number (001 – 0045). Ten 37.8 liter Aqueon® Glass Aquarium fish tanks were filled with deionized water and installed with Marina Power Filters (Slim S10). Additional DI water was added throughout the study period to ensure the aquaria were filled. Fifty grams/gal of Instant Ocean Aquarium Sea Salt Mixture were added to each of the 10 aquaria to reach a salinity of 15 ppt. Due to the experimental design, four of the experimental aquaria (two containing special concrete, one containing normal concrete, and one containing natural shell units; Figure 3.6) were on one side of the laboratory and five aquaria and the control (one containing special concrete, one containing normal concrete, one containing natural shell units; Figure 3.6) were on the other side of the laboratory. After one week, five shells from each treatment were randomly placed into

one of nine aquaria, with one aquarium as a control that contained no shells (Figure 3.6). After 12 weeks, all shells were removed from the aquaria.

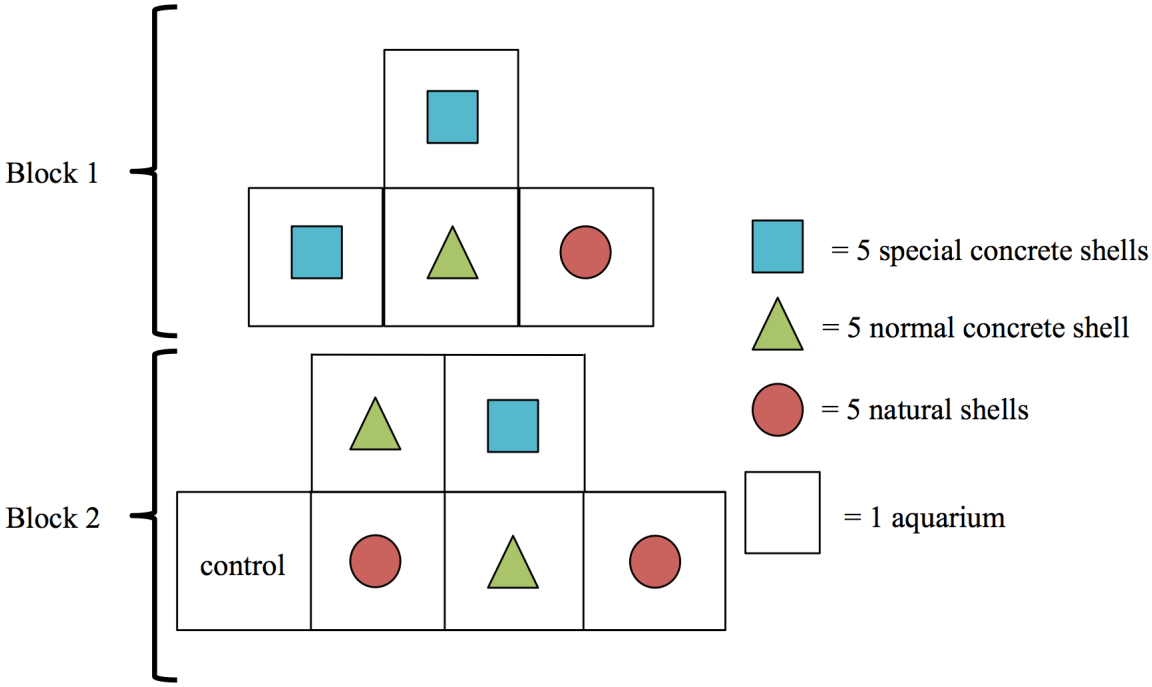

Figure 3.6. Experimental design. 15 units of each substrate (special concrete, normal concrete, natural shell) were randomly placed into 3 aquaria each resulting in 9 aquaria, each with 5 units of a different substrate. One aquarium had no substrate and was used as a control. There was an inadvertent effect of laboratory conditions on the experimental set up. Therefore, the blocks of the setup are designated and taken into account for the statistical analyses.

Water Chemistry Measurements

Several water chemistry measurements were taken throughout the study period: salinity, temperature, pH, conductivity, alkalinity, and calcium (Table 3.1). Salinity was amended bi-weekly by the manual addition of Instant Ocean Aquarium Sea Salt Mixture when measurements dropped below 10 ppt (Table A4). Every other day for 12 weeks, salinity (ppt), temperature (°C), pH (+/- 0.2 pH units), and conductivity (µS/cm; +/-3%) were measured using the Vernier LabQuest® 2 interface and the associated calibrated

sensors. Alkalinity (ppm $CaCO_3$; +/- 1 ppm) and calcium (ppm; +/- 1 ppm) measurements were taken once a week due to time and cost constraints by following the specifications of the Hanna Instruments Marine Alkalinity Checker® (HC – HI755) and Marine Calcium Checker® (HC – HI758), respectively.

Table 3.1. Water Quality Measurements Taken Water quality measurements were taken at different increments during the 12-week study period. Some of the measurements were response variables while others were used for monitoring.

Measurement	Response Variable or Monitored	Measurement Increment
Salinity (ppt)	Monitored	Every other day
Temperature (°C)	Monitored	Every other day
pH	Response Variable	Every other day
Conductivity (µS/cm)	Response Variable	Every other day
Alkalinity (ppm $CaCO_3$)	Response Variable	1/week
Calcium (ppm)	Response Variable	1/week

Water Collections

A one-liter sample of water from each aquarium (n = 10) was taken at three times throughout the study period: (1) immediately before shells were placed (Pre), (2) six weeks after shells were placed (Mid), and (3) twelve weeks after shells were placed (End). All water samples (n = 30) were sterile filtered using 0.2 micron Millipore® filters and stored in 1L Nalgene Wide Mouth Economy Bottles wrapped in tin foil and kept in a refrigerator for two to four months.

Alga Species Chosen

The algae strains used for this analysis are described in Table 3.2. Four axenic algae strains were received from Bigelow Laboratory and were prepared according to the

Bigelow culture methods (Bigelow – National Center for Marine Algae and Microbiota). The fifth algae strain, *Nannochloropsis,* was non-axenic and was cultured at James Madison University. Algae strains were chosen to represent a variety of types of algae found both in the Chesapeake Bay and around the world.

Aureococcus anophagefferens. A. anophagefferens is a marine alga that has been found along the entire eastern shore of the United States, including the Chesapeake Bay (Gobler et al. 2011). This species of algae is known to cause harmful algal blooms, specifically brown tides (Gobler et al. 2011), which can be toxic to bivalves. Brown tides typically form in shallow estuaries where oysters reefs are also known to inhabit.

Ostreococcus lucimarinus. Ostreococcus is a globally abundant phytoplankton genus and a member of the picoplankton community, which plays a central role in the ocean carbon cycle (Palenik et al. 2007). *O. lucimarinus* has adapted to thrive in high light intensity conditions, and therefore is found on the surface of the water (Palenik et al. 2007).

Emiliania huxleyi. E. huxleyi is a globally abundant species of coccolithophore and plays a major role in the base of marine food webs (Holligan et al. 1993, Pond and Harris 1996). Like all coccolithophores, *E. huxleyi* produces external plates that are made of calcium carbonate (Holligan et al. 1993). Therefore, just like oysters, *E. huxleyi* may be affected by OA and the reduced concentration of calcium carbonate in the water column.

Thalassiosira pseudonana. T. pseudonana is a marine diatom that can be found widely distributed in the world's oceans and can account for about 20% of the world's carbon fixation (Armbrust et al. 2004). It was important that a variety of alga species were tested, including diatoms because they are globally distributed.

Nannochloropsis. The *Nannochloropsis* genus consists of 5 known marine species that are regularly used as a food source in marine aquaculture (Krienitz and Wirth 2006). Therefore, this genus is important for food web interactions, especially on oyster reefs.

Table 3.2. Algae Strains Chosen for Study Five algae strains were used in this analysis. All strains are from Bigelow Laboratory, except *Nannochloropsis*.

Strain	Species	Typical Range	Axenic	Common Name
CCMP 1984	*Aureococcus anophagefferens*	Coastal	Yes	Chromista
CCMP 3430	*Ostreococcus lucimarinus*	Global	Yes	Chlorophyta
CCMP 374	*Emiliania huxleyi*	Global	Yes	Coccolithophore
CCMP 1015	*Thalassiosira pseudonana*	Global	Yes	Centric diatom
Nano	*Nannochloropsis* sp.	Global	No	

Media and Algae Inoculation

All Pre water samples (n = 10) were pooled together because all aquaria had the same contents (DI water + 600g Instant Ocean; Table 3.3). One hundred ml from each Pre water sample were combined to make the Pre media. The replicates within each treatment (natural shell, normal concrete, special concrete) from the Mid and End water samples were pooled to ensure the results were dependent on the treatment (shell type) and not on the variation between aquaria within each treatment (Table 3.3; Figure 3.7). The same was done for the End water samples (n = 10) to create the End media for each

treatment. Nutrients were aseptically added to the pooled media to create L1 or L1 + Si media (Figure 3.7; Guillard and Hargraves 1993). For the control treatments, nutrients were aseptically added directly to the 1L water sample taken at either the Mid or End time point to create L1 or L1 + Si media (Table 3.3; Guillard and Hargraves 1993). Thirty ml were aseptically dispersed into tubes (n = 135), and the media was aseptically inoculated with the appropriate algae strain (n = 5). Each algal strain was grown in triplicate.

Table 3.3. Media Development The media for algae inoculation were made using the water samples taken at the three time points (Pre, Mid, End) of the study period. All Pre water samples were pooled, the control samples were unchanged, and the 3 samples from 1 treatment were pooled at the different sample times (Mid, End) to reduce variation within treatments. A total of 9 medias were made from the initial 30 water samples. The 9 medias were used to create 15 tubes for algae inoculation of 5 strains each (5 strains done in triplicate).

Treatment and Sample Time	Number of Samples	Media Made	Tubes Inoculated
All treatments- Pre	10	1	15
Control- Mid	1	1	15
Natural Shell- Mid	3	1	15
Normal Concrete- Mid	3	1	15
Special Concrete- Mid	3	1	15
Control- End	1	1	15
Natural Shell- End	3	1	15
Normal Concrete- End	3	1	15
Special Concrete- End	3	1	15
Total	**30**	**9**	**135**

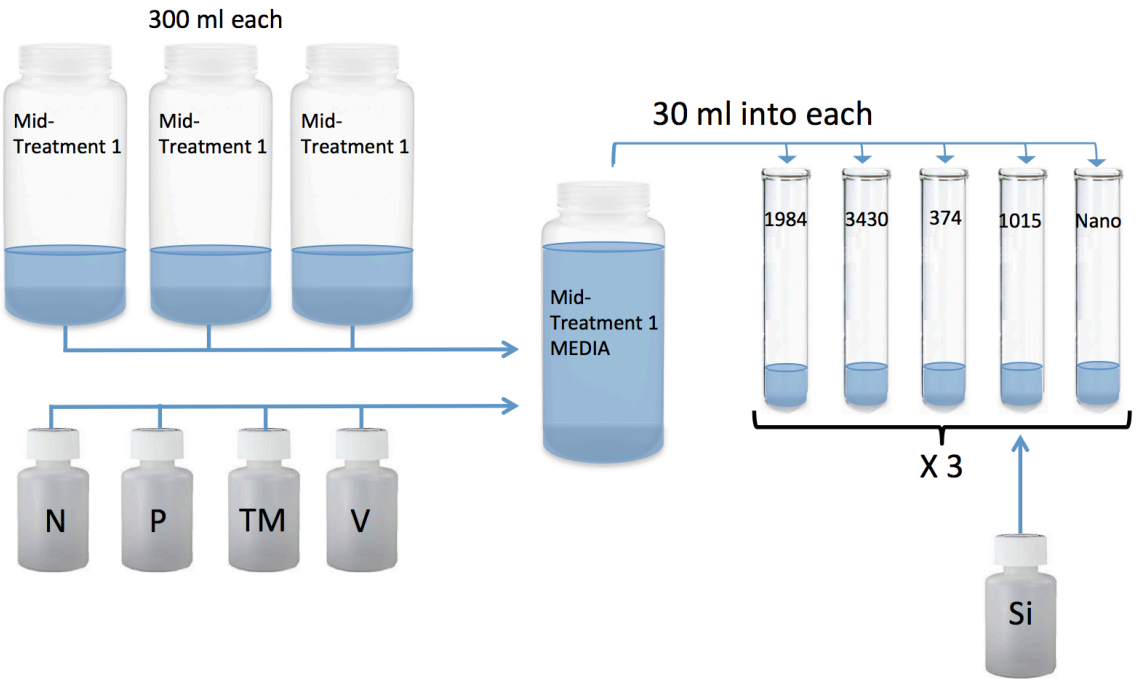

Figure 3.7. Media Creation The above diagram demonstrates the sequence of events for the Mid and End water samples. The three replicates of each treatment were pooled together. To finish the media, Nitrogen (N), Phosphorus (P), Trace Metals (TM), and Vitamins (V) were aseptically added to the pooled water samples. Once the media was made, 30ml was aseptically added to tubes that would contain one of 5 algal strains. Each algal strain was done in triplicate. Silica (Si) was added only to the 1015 strain because it was the only diatom. 1984= *Aureococcus anophagefferens*, 3430= *Ostreococcus lucimarinus*, 374= *Emiliania huxleyi*, 1015= *Thalassiosira pseudonana*, Nano= *Nannochloropsis.* The Pre water treatment had 10 water samples with 100ml of each in the media. Nutrients (N, P, TM, and V) were aseptically added directly to the Mid (n =1) and End (n = 1) control water samples without pooling. See table 3 for details about what water samples went into the media. Curves for fluorescence and Fv/FM were placed on the same plot. One plot per strain (n = 5) per treatment (n = 9) was generated.

Measurements

Fluorescence. Fluorescence measurements were taken at the same time every other day until a typical growth curve was reached and measurements began to level off in the Pre treatment. To do this, 2 ml of sample was placed into a cuvette and put into the WALZ PHYTO-PAM-II Compact Version Phytoplankton and Photosynthesis Analyzer (Effeltrich, Germany). Measurements for all wavelengths (440nm, 480nm, 540nm,

590nm, and 625nm) were recorded but only the average (n = 3) of the 480nm measurements were used for analysis.

Fv/Fm. Fv/Fm measurements were recorded at the same time as the fluorescence measurements. All tubes (n = 135) were kept in an incubator at 19°C on a 14:10 light:dark cycle. For measurements, all tubes were dark adapted for 30 minutes prior to analysis and kept in the dark until the measurements were recorded. The same 2ml sample from above was used to record Fv/Fm. Measurements for all wavelengths (440nm, 480nm, 540nm, 590nm, and 625nm) were recorded but only the average (n = 3) of 480nm measurements were used for analysis.

Statistical Analysis

Water Quality. The purpose of this study was to determine if there was an effect of treatment (natural shell, normal concrete, and special concrete) on water quality parameters chosen for this study (pH, conductivity, alkalinity, and calcium). Each treatment had 3 replicates that were pooled to increase sample size.

There was an initial conditioning period for the treatments in all of the aquaria between September 6, 2016 and October 4, 2016. During this period, there was high variation in all aquaria for all treatments, which ended with a clear stabilization when plotted. Therefore, measurements taken between those dates were not included in the analysis. To ensure there were no missing data, only the dates in which all 6 parameters were measured from October 4, 2016 to November 29, 2016 were included in the analysis (n = 8; Table A3). The distributions of pH, conductivity, alkalinity, and calcium

values were evaluated using Shapiro-Wilk tests and the associated Q-Q Plots. The distributions of conductivity and calcium measurements for all treatments were normal according to the Shapiro-Wilk test ($p > 0.05$). Two treatments (special concrete and normal concrete) for pH and 1 treatment for alkalinity (normal concrete) failed the Shapiro-Wilk test for normality ($p < 0.05$). However, the sample sizes ($n = 24$; 8 measurements with 3 replicates each) were not large enough to categorize these measurements as being non-normally distributed. Small sample sizes are much more sensitive to outliers than large sample sizes. Therefore, a parametric test was used.

Because of this design, there was an inadvertent block effect caused by varied laboratory conditions (MANOVA; $p < 0.001$). A Type II Sum's of Squares ANOVA model was created for all parameters measured to test for an interaction between treatment and block. A typical Type I ANOVA was not used because Type II is more appropriate for unbalanced experimental designs (four aquaria on one side and six on the other). There was not an interaction between treatment and block (pH; $p = 0.107$, conductivity; $p = 0.547$, alkalinity; $p = 0.5766$, calcium; $p = 0.091$), so the interaction term was removed from the model resulting in Type II two-way ANOVA's to test for the effects of treatment and block on the water quality parameters measured. A Tukey Honest Significant Differences (HSD) test was used to determine the specific differences in treatments. All statistical analyses were completed in RStudio (Version 1.1.136; Appendix II).

Fluorescence and Fv/Fm. These data resulted in repeated measures curves over time; therefore, qualitative investigations were completed instead of a quantitative statistical

analysis. The fluorescence curves between treatments were visually compared for each algae strain. In addition, the Fv/Fm curves were also visually compared.

Results

Aquaria Measurements

Temperature and salinity were monitored throughout the 12-week study period. The temperature of all tanks averaged 22.3 °C (SD = 0.53 °C) and salinity of all aquaria remained between 10 and 15 ppt ($\bar{x}$ = 13.69, SD = 1.99). These values are typical values seen in Pitman Cove in Kilmarnock, VA where the oyster larval recruitment study was conducted.

For pH values, there was a significant effect of both treatment (ANOVA; $p < 0.001$) and block (ANOVA; $p < 0.001$; Table 3.4). For conductivity, there was no effect of treatment (ANOVA; $p = 0.182$) or block (ANOVA; $p = 0.996$; Table 3.4). For alkalinity, there was a significant effect of treatment (ANOVA; $p < 0.001$) but not block (ANOVA; $p = 0.873$; Table 3.4). Finally, for calcium, there was a significant effect of both treatment (ANOVA; $p = 0.035$) and block (ANOVA; $p < 0.001$; Table 3.4).

Table 3.4. ANOVA Results Type II Two-way ANOVA results for each response variable and the main effects. The interaction between treatment and block was not significant for any response variable and was removed from the model.

Response Variable	dF	F statistic	p value
pH			
Treatment	3	59.9	< 0.001 ***
Block	1	132.4	< 0.001 ***
Conductivity			
Treatment	3	1.6	0.182
Block	1	0.0	0.995
Alkalinity			
Treatment	3	39.5	< 0.001 ***
Block	1	0.025	0.872
Calcium			
Treatment	3	3.01	0.035 *
Block	1	27.5	< 0.001 ***

* Signifies $p < 0.05$ *** Signifies $p < 0.001$.

pH. There was a significant effect of both treatment and block for pH values in the 10 aquaria. Post-hoc comparisons show that each combination of treatments was statistically significant from each other (Tukey HSD; all $p < 0.05$; Figure 3.8). The special concrete treatments had significantly lower pH levels (8.27) than normal concrete (8.32; Tukey HSD; $p = 0.0015$), natural shell (8.45; $p < 0.001$), and the control aquaria (8.51; $p < 0.001$; Figure 3.8). The pH levels of the normal concrete treatment were significantly lower than natural shell ($p < 0.001$) and the control ($p < 0.001$; Figure 3.8) aquaria. The natural shell pH levels were significantly lower than the control aquaria ($p = 0.003$; Figure 3.8). Finally, all aquaria in Block 2 had significantly higher pH levels than aquaria in Block 1 ($p < 0.001$; Figure 3.8). Since there was no interaction between

treatment and block, I can assume the increases in pH from Block 1 to Block 2 for all treatments was proportional.

Conductivity. There was not a significant effect of treatment ($p = 0.182$) or block ($p = 0.996$; Table 3.4) on the conductivity measurements (μS/cm) in any aquaria (Figure 3.8). Conductivity values ranged from 4320 μS/cm to 4520 μS/cm for the special concrete treatment ($\bar{x}$ = 4423 μS/cm), 4344 μS/cm to 4548 μS/cm for the normal concrete treatment ($\bar{x}$ = 4453 μS/cm), and 4342 μS/cm to 4522 μS/cm for the natural shell treatment ($\bar{x}$ = 4427 μS/cm).

Alkalinity. There was a significant effect of treatment ($p < 0.001$), but not block ($p = 0.872$; Table 3.4; Figure 3.8), on alkalinity measurements. Post-hoc comparisons reveal there was not a significant difference between the special concrete and normal concrete treatments ($p = 0.99$; Figure 3.8). However, the special concrete aquaria had lower alkalinity (133.3 ppm) than natural shell (196.3 ppm; $p < 0.001$) and the control aquaria (213.6 ppm; $p < 0.001$; Figure 3.8). In addition, there was no significant difference in alkalinity between the natural shell and control aquaria ($p = 0.401$; Figure 3.8).

Calcium. The Type II Two-way ANOVA revealed a significant effect of treatment ($p = 0.035$) on calcium levels (ppm). However, post-hoc comparisons did not reveal any significant differences between treatments (all $p > 0.05$; Figure 3.8). The significant effect that the ANOVA revealed for treatment is most likely due to the strong significance of block on calcium levels ($p < 0.0001$; Figure 3.8). Block 2 had

significantly lower calcium levels (474.9 ppm) than Block 1 (502.6 ppm; $p < 0.001$; Figure 3.8).

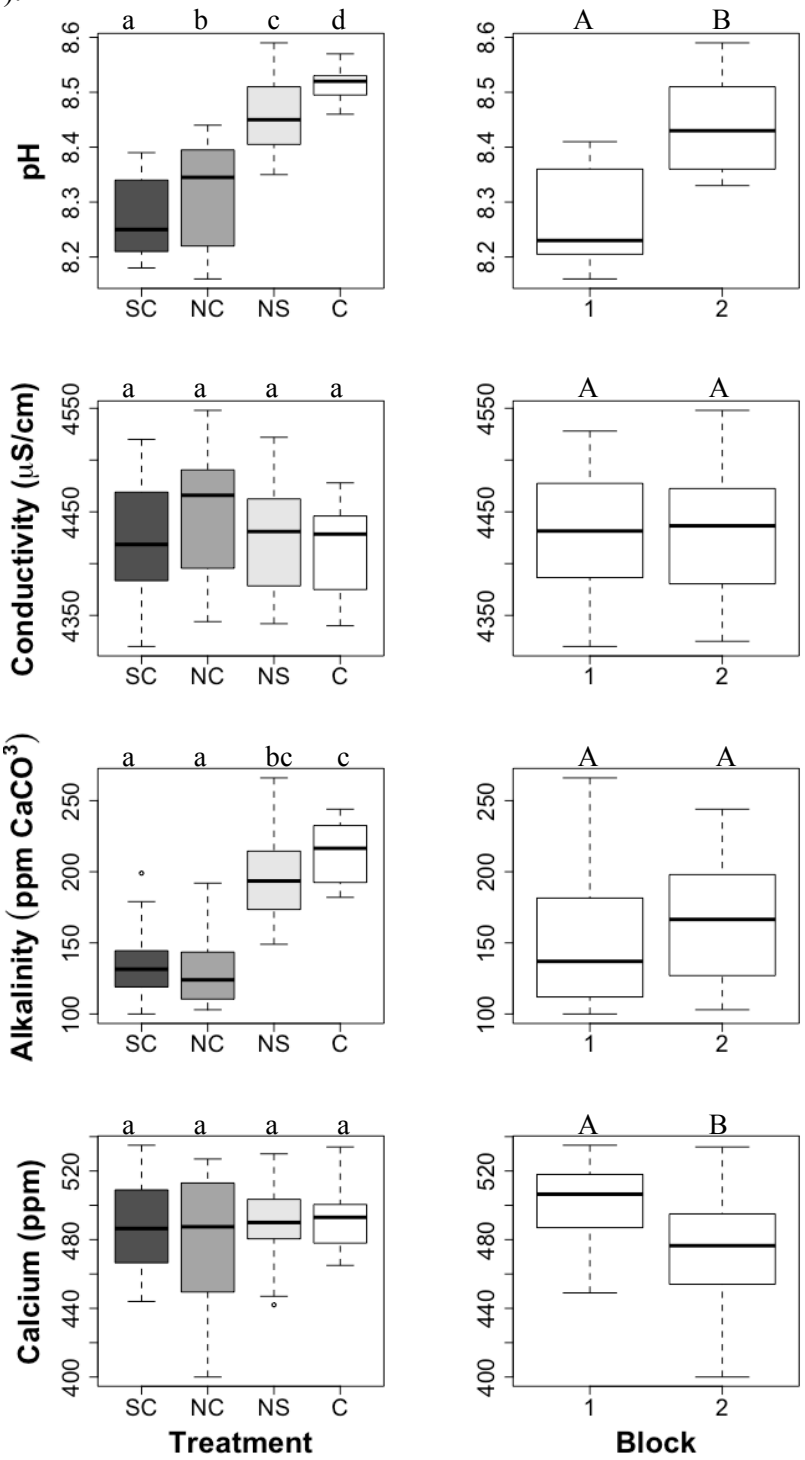

Figure 3.8. ANOVA Results Boxplots and ANOVA results reveal significant differences between treatments and block for some response variables, but not others. Lowercase letters indicate significant differences between treatments. Uppercase letters indicate significant differences between blocks. SC = special concrete, NC = normal concrete, NS = natural shell, C = control.

Algae Growth

Fluorescence. All algae strains (n = 5) had successful fluorescence (proxy for growth), but not all treatments (n = 9) supported the growth of every strain, suggesting a treatment effect on growth. *A. anophagefferens* (strain CCMP 1984) was the least successful strain with the least amount of growth in the least amount of treatments (min = -10.6, max = 86.6; Figure 3.9). *O. lucimarinus* (strain CCMP 3430; Figure 3.10) and *E. huxleyi* (strain CCMP 374; Figure 3.11) grew in all treatments, but at varying levels (min = -10, max = 35.6; min = -9.6, max = 96.6, respectively). *T. pseudonana* (strain CCMP 1015; Figure 3.12) grew relatively consistently in all treatments (min = -9, max = 230.3). Finally, *Nannochloropsis* had the greatest and most consistent growth across all treatments (min = -8, max = 331.3; Figure 3.13).

Fv/Fm. Fv/Fm is a measure of cell stress and is calculated by looking at the difference between maximum fluorescence and minimum fluorescence. Cells that are more stressed have lower values of Fv/Fm.

Similar to fluorescence, all strains showed levels of stress, but the stress varied between strains and treatments, again suggesting an effect of treatment on stress levels. There was growth of *A. anophagefferens* (strain CCMP 1984) in some treatments, but consistently low Fv/Fm values indicate cell stress despite that growth (min = 0, max = 0.37; Figure 3.9). *O. lucimarinus* (strain CCMP 3430) and *E. huxleyi* (strain CCMP 374) had inconsistent cell stress across treatments (min = 0, max = 0.35; min = 0, max = 0.4, respectively; Figure 3.10 and 3.11). *T. pseudonana* (strain CCMP 1015) grew in all treatments but some treatments created higher stress levels than others (min = 0, max =

0.44; Figure 3.12). Finally, *Nannochloropsis* had the least and most consistent stress across all treatments (min = F0, max = 0.58; igure 3.13).

Curve Comparisons of Fluorescence and Fv/Fm Between Treatments

Aureococcus anophagefferens. Strain CCMP 1984 had the least amount of growth for all treatments, except the End time control treatment. The Pre- water treatment showed no growth, and therefore, stress could not be measured (Figure 3.9). The special concrete treatments showed no growth. The normal concrete treatments showed very little growth at the end of the study period, but a slight leveling off can already be seen, suggesting no new growth would have occurred with a longer study period. The natural shell treatment showed a similar pattern to the normal concrete treatment, with a drop in fluorescence towards the end of the study period. The two control treatments (Mid and End) showed growth halfway through the study period. The Mid control treatment had an eventual leveling off by the end of the study period with no measurements of stress. The End control treatment showed high levels of fluorescence with moderate levels of cell stress (Figure 3.9).

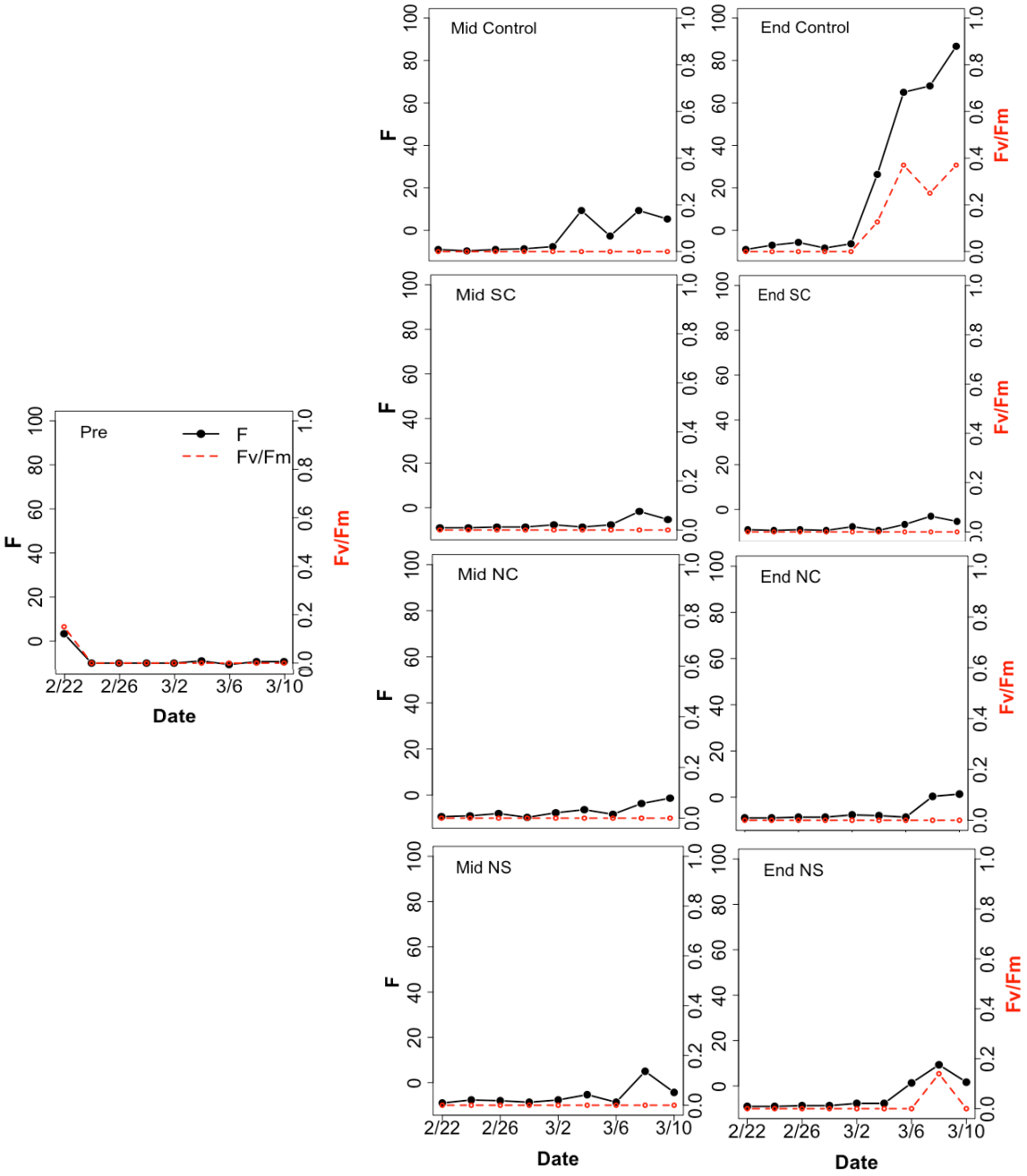

Figure 3.9. F and Fv/Fm for Strain CCMP 1984 Fluorescence (proxy for growth) and Fv/Fm (proxy for stress) measurements for *Aureococcus anophagefferens* between February 22 and March 10, 2017. Each graph depicts the florescence and Fv/Fm measurements in a different water treatment. Higher F measurements indicate greater growth. Lower Fv/Fm measurements indicate greater stress.

Ostreococcus lucimarinus. Strain CCMP 3430 grew in all treatments, but at varying levels. In addition, some treatments created greater cell stress than other treatments (Figure 3.10). All treatments supported less growth and higher stress levels than the Pre treatment. The Pre treatment showed little stress with more growth as time progressed. The special concrete treatments supported minimal growth, with no measurements of stress available. The normal concrete treatments supported greater growth than the special concrete treatment with consistent stress levels for the Mid and End time points. Natural shell treatments also supported growth over time, but there were differences in cell stress levels. The Mid natural shell treatment showed lower stress levels than normal concrete, but the End natural shell treatment showed high stress levels despite growth (Figure 3.10). The Mid control treatment supported growth and had a stress level similar to both normal concrete treatments while the End control treatment supported similar growth and stress levels as the special concrete treatments (Figure 3.10).

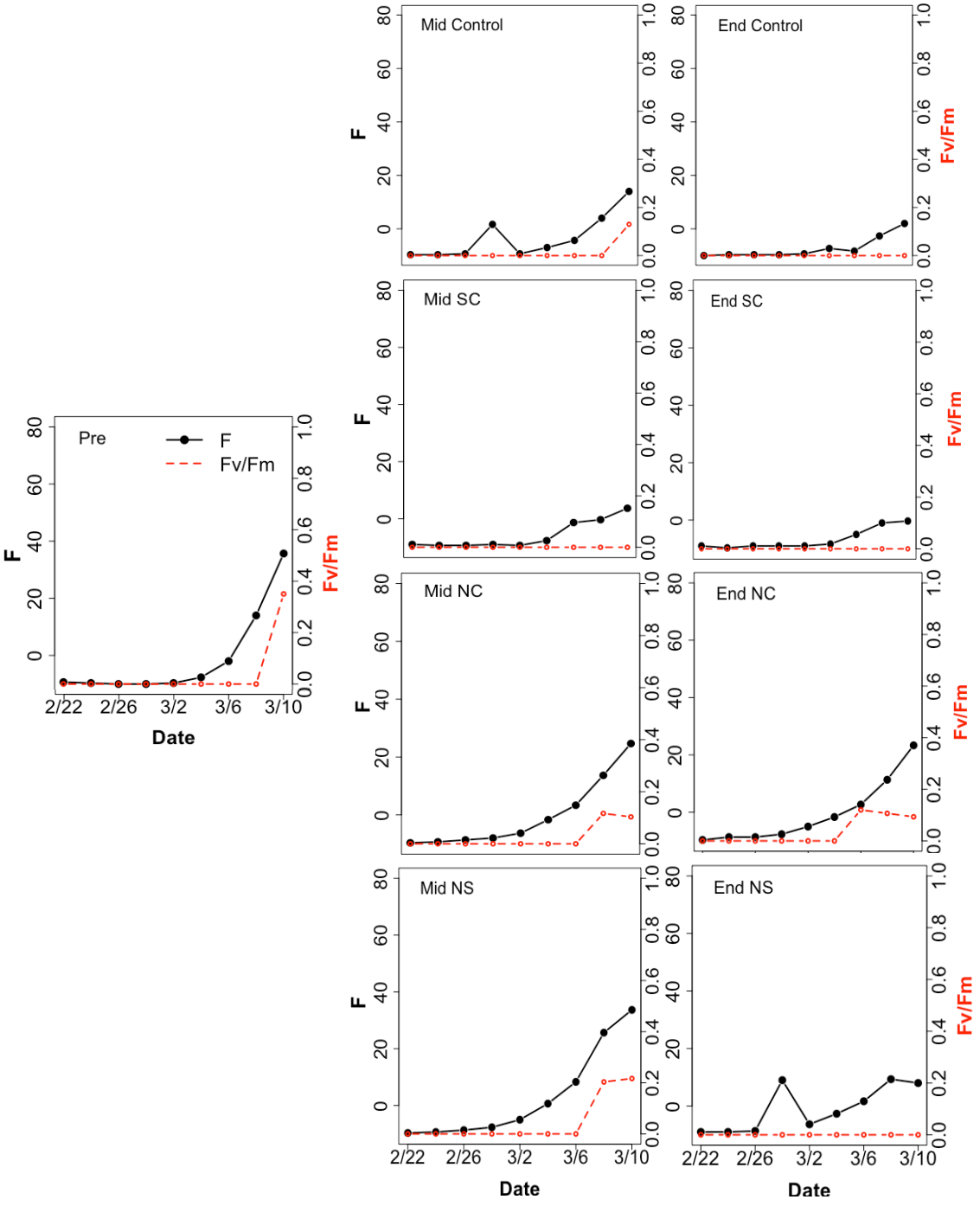

Figure 3.10. F and Fv/Fm for Strain CCMP 3430 Fluorescence and Fv/Fm measurements for *Ostreococcus lucimarinus* between February 22 and March 10, 2017. Each graph depicts the florescence and Fv/Fm measurements in a different water treatment.

Emiliania huxleyi. Similar to *O. lucimarinus,* strain CCMP 374 grew in all treatments and had varying stress levels. The Pre treatment supported eventual growth with lower stress levels as time progressed (Figure 3.11). The special concrete treatments supported minimal growth throughout the study period. The Mid normal concrete treatment supported similar growth and stress levels as the Pre treatment, while the End normal concrete showed similar growth and stress levels as the special concrete treatments. The natural shell treatment supported similar growth and stress levels as the Mid normal concrete treatment. The Mid control treatment supported the greatest growth and lowest stress levels of all treatments. The End control treatment did not show a normal growth curve possibly due to improper mixing of the solution prior to sampling and measuring. Therefore, the results of that specific treatment are not reliable (Figure 3.11).

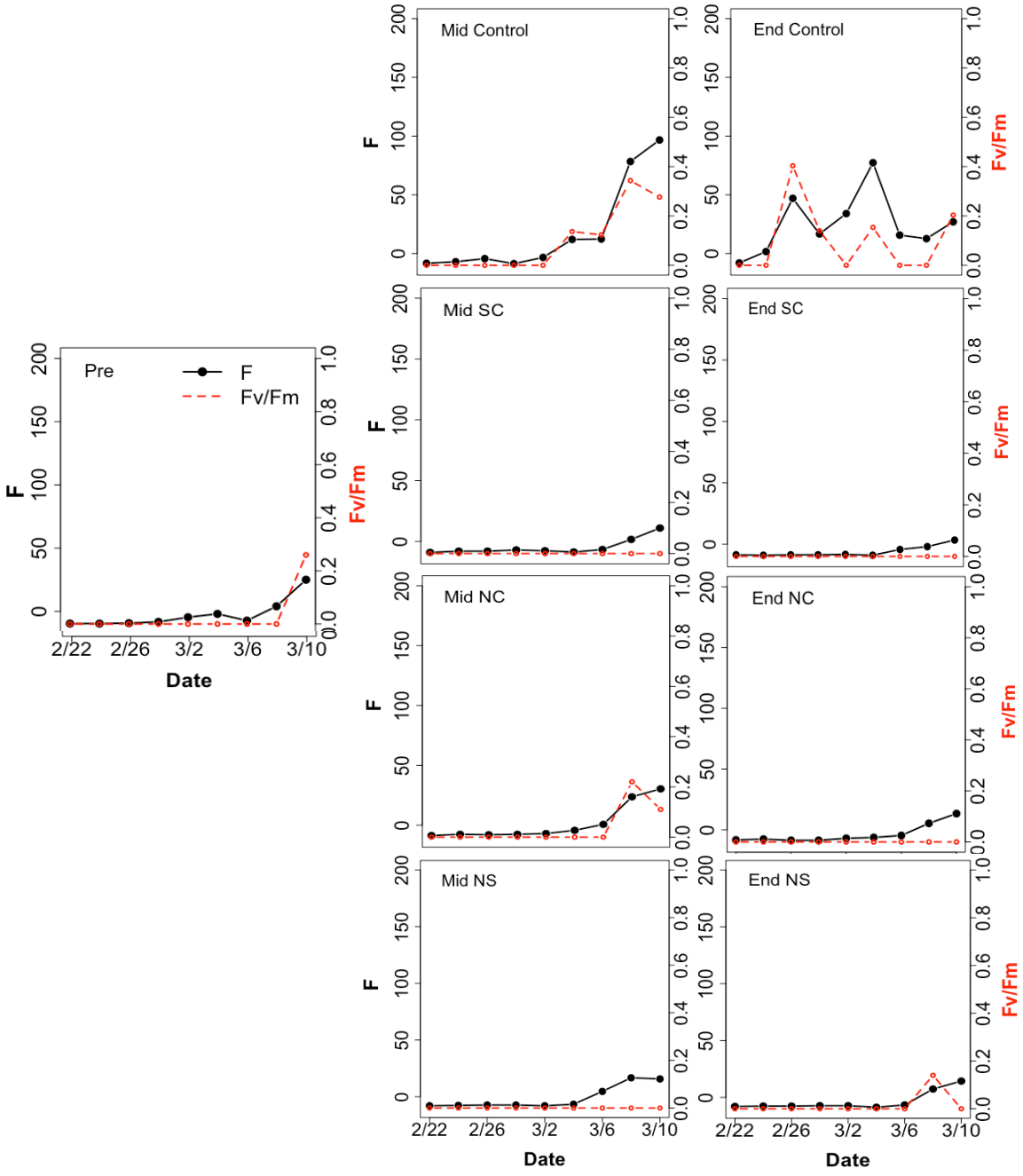

Figure 3.11. F and Fv/Fm for Strain CCMP 374 Fluorescence and Fv/Fm measurements for *Emiliania huxleyi* between February 22 and March 10, 2017. Each graph depicts the florescence and Fv/Fm measurements in a different water treatment.

Thalassiosira pseudonana. Strain CCMP 1015 had similar stress levels across all treatments with varying levels of growth over time (Figure 3.12). The Pre treatment supported the greatest amount of growth with consistent stress levels as time progressed. The special concrete, normal concrete, and natural shell treatments supported similar levels of growth with consistent stress levels. The control treatments supported greater growth than the special concrete, normal concrete, and natural shell, but lower growth than the Pre treatment. Again, the stress levels for the control treatment were similar to all other treatments (Figure 3.12). In all treatments, except Pre, Mid control, and End normal concrete, there was a sharp decrease in fluorescence on March 2, but a subsequent increase on the following measurement (March 4). This can be attributed to improper mixing of the solution prior to taking measurements.

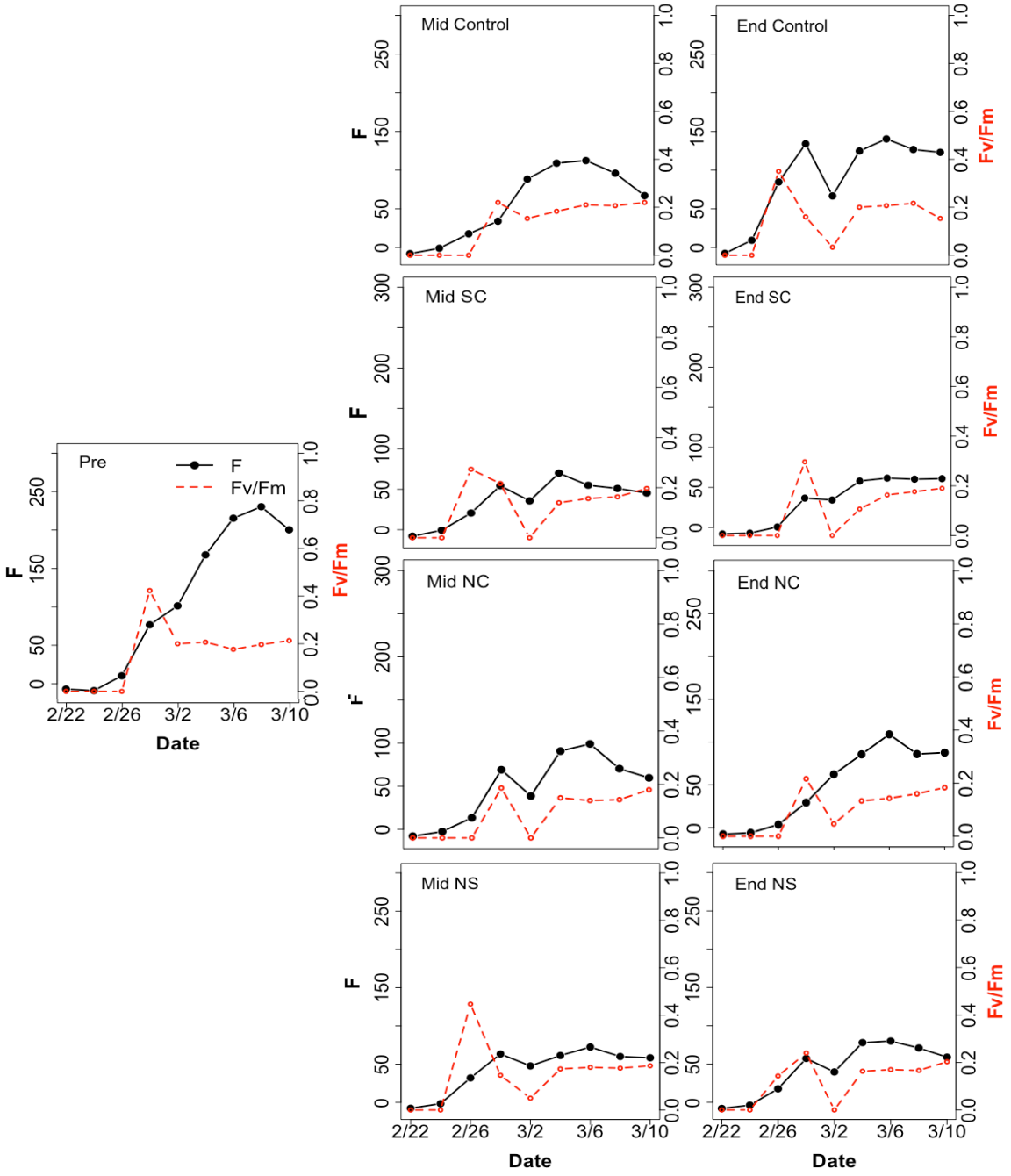

Figure 3.12. F and Fv/Fm for Strain CCMP 1015 Fluorescence and Fv/Fm measurements for *Thalassiosira pseudonana* between February 22 and March 10, 2017. Each graph depicts the florescence and Fv/Fm measurements in a different water treatment.

Nannochloropsis. *Nannochloropsis* (Nano) showed similar growth and stress patterns across all treatments (Figure 3.13). The Pre treatment supported the greatest growth, followed closely by both control treatments. The special concrete, normal concrete, and natural shell treatments supported slightly less growth, but stress levels were still consistent with the Pre treatment (Figure 3.13).

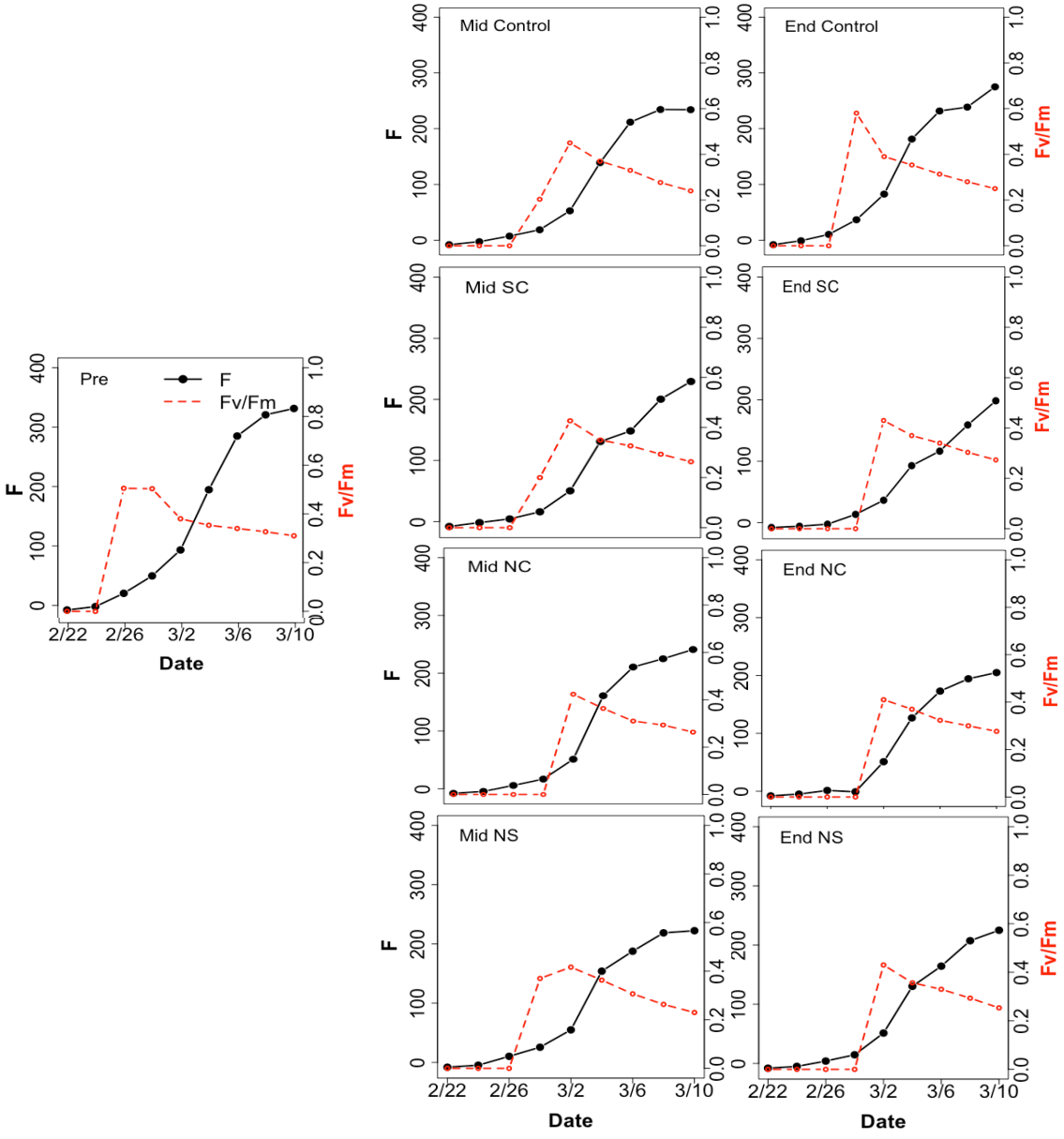

Figure 3.13. F and Fv/Fm for *Nannochloropsis* Fluorescence and Fv/Fm measurements for *Nannochloropsis* between February 22 and March 10, 2017. Each graph depicts the florescence and Fv/Fm measurements in a different water treatment.

Discussion

The goal of this study was to assess the effects of an artificial substrate with augmented levels of calcium on localized water quality as well as the effect on subsequent algal growth to determine the quality of this substrate for oyster restoration. To test this, I exposed artificial seawater (DI water and Instant Ocean Aquarium Sea Salt Mixture) to special concrete, normal concrete, and natural oyster shell units. Several water quality parameters were measured (pH, conductivity, alkalinity, and calcium) to determine if the substrates had any negative effect after a 12-week study period. In addition, water samples were taken from the aquaria holding the substrates before (Pre), midway (Mid), and the end (End) of the 12-week study period. The water samples were then used to grow a suit of algae species known to occur in the Chesapeake Bay to determine if the substrates were releasing harmful elements that would prevent algal growth, suggesting a negative effect on the base of the food web on oyster reefs.

There was an effect of treatment on pH and alkalinity with the special concrete having lowered values compared to the other treatments. This did not support our initial prediction, but upon further investigation, the values seen here were not biologically significant. There was no effect of treatment on conductivity or calcium levels, which did support our prediction. In addition, there was a slight decrease in growth of *O. lucimarinus* and *E. huxleyi* in some treatments, however this effect is not dramatic. *T. pseudonana* and *Nannochloropsis* showed similar growth despite treatment. Finally, *A. anophagefferens* showed the least amount of growth despite treatment. These results support our prediction that the treatments would not have a great effect on the growth of the algal strains chosen.

Aquaria Measurements

pH. Contrary to what I predicted, the pH of the aquaria with special concrete (8.27) units was statistically significantly lower than any other treatment, followed by normal concrete (8.32), natural shell (8.45), and finally the control aquarium with the highest pH (8.51). There was a block effect present, but the interaction between treatment and block was not significant. Therefore, the significantly greater pH in Block 2 is just a proportional increase in pH for all treatments from Block 1.

Although the values of pH for each treatment were statistically significant from each other, they potentially hold no biological significance. In natural environments, the average pH of oceanic waters is 8.1 (Porzio et al. 2011). All values of pH (8.16-8.59) in the experimental aquaria were above this global average. It is only at lower pH values where there is a significant effect on the dissolution of calcium carbonate shells and reproductive impairment (Kurihara and Ishimatsu 2007, Talmage and Gobler 2010, Waldbusser et al. 2011, Waldbusser et al. 2011). Therefore, the negative effect that the special concrete treatments seemed to have on pH is not biologically significant for this system. In addition, the accuracy of the pH probe was +/- 0.2 pH units, which is within the range of values seen in this study, suggesting the differences seen might not be valid.

Conductivity. There was no effect of treatment, or block, on conductivity levels in any aquaria (4320 µS/cm to 4548 µS/cm). Conductivity is a measure of how well a body of water can transmit electricity. For seawater, this refers to the concentration of ions in the water (Miller et al. 1988). Ions in seawater come from dissolved salts as well as carbonate compounds (EPA 2012). Therefore, the more saline the water is, the higher

conductivity it has. Conductivity is the most commonly measured water quality parameter and is a reliable early indicator of changes in a water system (Miller 1988). A change in the conductivity of water can signify ions being added and a potential indicator of pollution in the system (EPA 2012).

What is promising in this study is that there was no significant difference of conductivity between any of the treatments. Therefore, this suggests that none of the treatments were off-putting additional ions into the surrounding environment. This piece of evidence supports the prediction that the special and normal concrete treatments would not cause harm to their surrounding environment. In addition, these data suggest that the addition of artificial substrates for restoration would not cause immediate harm to their surrounding environment.

Alkalinity. The aquaria with the special concrete treatment had statistically significantly lower alkalinity values than the aquaria with the natural shell and control treatments. However, these values were not statistically different from normal concrete, supporting our prediction.

Alkalinity is a measure of how well a body of water can resist changes in pH. For seawater, it is typically referred to as the amount of carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-) ions present (Kaushal et al. 2013). Both of these ions are able to accept free hydrogen ions, effectively removing the acidity-causing ion from the water, and help resist any further changes in pH. Lower values of alkalinity signify reduced ability to buffer changes in pH. However, similarly to the pH values measured, the alkalinity values measured for the aquaria with the concrete treatments are within the range of

normal seawater (100-130 ppm $CaCO_3$). The aquaria with the natural shell treatment had increased alkalinity (196 ppm $CaCO_3$) suggesting the addition of calcium carbonate oyster shells has the ability to increase the surrounding water's ability to neutralize any changes in pH.

Calcium. The Type II Two-way ANOVA revealed a significant effect of treatment on calcium levels; however, post-hoc comparisons showed there was no significant difference between treatments. The significant ANOVA is most likely due to the highly significant block effect seen. Despite the block effect, there was no difference in calcium levels in any of the treatments. This result suggests that none of the substrates were releasing additional calcium into the surrounding environment at concentrations that were detectable. This is further supported by the non-significant differences seen in conductivity between treatments. Calcium is a cation that would have been detected in the conductivity measurements if it were present.

With OA, there is less available calcium carbonate in the water column, leaving calcifying organisms with fewer resources to build their shells (Riebesell et al. 2000). I predicted that calcium would be released by the special concrete treatment into the surrounding environment. The dissolution of calcium into the water had the possibility to provide local calcifying organisms with the buildings blocks they need to make their shells. However, this did not occur potentially for a couple reasons. This could be due to the short duration of the study. With more time, the special concrete could dissolve more, releasing calcium into the surrounding environment. In addition, the breakdown of

concrete in saltwater increases with higher temperature and mechanical damage possibly due to high wave action (Mather 1964)

Algae Growth

Fluorescence. All strains had successful growth, but the success varied by treatment and strain. All strains grew the most in the Pre treatment water. One reason for this could be due to a room effect over time. The Pre water samples were taken at the start of the 12-week period when the treatment was only DI water and Instant Ocean Aquarium Sea Salt Mixture. Over time, dust and other elements could have gotten into the aquaria due to the aquaria being open to the room at all times. The addition of foreign elements in addition to the experimental units could have negatively affected the growth of the algae strains chosen for this analysis. Another reason for why the Pre treatment potentially supported the most algal growth across strains is because minor dissolution of experimental units could have affected algal growth. Particles not detected by the water quality measurements that were released by the shell units could have made the water unfavorable for supporting algae growth.

The most useful comparison to make with each strain is comparing the growth of algae in the natural shell treatment against growth in the concrete treatments. Since oyster shells are a natural substrate that is present in the ocean, it can be assumed that algae can naturally grow in the presence of this substrate. Therefore, any differences in algal growth between the natural shell treatment and the concrete treatments were most likely due to the difference in treatment.

A. anophagefferens (strain CCMP 1984) took the longest to start growing and had minimal growth in the concrete and natural shell treatments. The minimal growth of this strain may be perceived as positive. *A. anophagefferens* is known to cause brown tides which can be toxic to bivalves. The fact that this strain did not grow in the Pre treatment and had minimal growth in the concrete and natural shell treatments suggests that something besides the treatments, potentially the salinity, was preventing growth. However, it is unclear why this strain grew in the End control treatment.

O. lucimarinus (strain CCMP 3430) had successful growth in all treatments. The growth in the natural shell treatment and normal concrete treatment were very similar suggesting no adverse effects caused by the normal concrete. The special concrete treatment supported slightly lower growth of *O. lucimarinus* than both the normal concrete and natural shell treatments. This suggests that the special concrete treatment has a small adverse effect on the growth of this one strain of phytoplankton. Other phytoplankton strains would also have to be examined to determine if this effect is consistent.

E. huxleyi (strain CCMP 374) growth in all treatments was similar to *O. lucimarinus.* Again, the normal concrete and natural shell treatments supported similar growth, with the special concrete treatment supporting slightly lower growth. Initially, the lower growth was thought to be a consequence of the lower pH found in the special concrete aquaria because coccolithophores are calcifying organisms. *E. huxleyi* could be more sensitive to pH and the lower pH in the special concrete treatments could have led to lower growth. (Gao et al. 2009). However, this is contrary to what has been found in other work (Iglesias-Rodrigues et al. 2008, Shi et al. 2008, Beaufort et al. 2011). Similar

to *O. lucimarinus*, additional species of coccolithophores need to be examined to determine if the special concrete treatment displays the same patterns with lower growth.

T. pseudonana (strain CCMP 1015) showed comparable growth across the concrete and natural shell treatments. There does not seem to be any obvious effect of treatment on the growth of this strain of diatom. This result is promising because diatoms are known to play an important role in carbon fixation (Armbrust et al. 2004). Placing an artificial substrate that would prevent the growth of a species of diatom that is beneficial to combating OA would not be beneficial to the surrounding environment. Additional diatom species would also need to be examined to determine if the pattern of no affect holds.

Nannochloropsis was expected to grow regardless of treatment because of the way it was cultured. This was the only strain that was not axenic. This means that it was cultured in the presence of other bacteria and has the ability to grow despite competition from bacteria. Therefore, it was not surprising that *Nannochloropsis* had similar growth in all treatments. In natural environments, other strains of algae will not be axenic and will likely grow similarly to *Nannochloropsis.*

Fv/Fm. There was varying cell stress levels between strains as well as between treatments within strains. For some treatments and strains, there was minimal growth, which prevented stress measurements from being recorded. This was the case for *A. anophagefferens* (strain CCMP 1984). There was minimal growth in most treatments and, therefore, stress levels could not be determined.

O. lucimarinus (strain CCMP 3430) showed similar stress levels between the normal concrete and natural shell treatments. Because of the minimal growth of *O. lucimarinus* in the special concrete treatment, stress levels could not be determined. I can conclude that the *O. lucimarinus* growing in the normal concrete and natural shell treatments were not stressed due to similar Fv/Fm values, but no conclusions can be made about *O. lucimarinus* growing in the special concrete treatment.

E. huxleyi (strain CCMP 374) showed a similar pattern of growth and stress in the concrete and natural shell treatments. Minimal growth led to minimal stress measurements. Again, no solid conclusions can be made about the stress of the cells growing.

T. pseudonana (strain CCMP 1015) showed very similar stress levels between treatments. There is one consistent drop in the Fv/Fm measurement on March 2, 2017. This is most likely not due to an increase in stress levels, but rather an improper mixing of the sample before taking a measurement. The drop in fluorescence that coincides with the drop in Fv/Fm reinforces this hypothesis. By not mixing the sample before removing 2ml for the measurement, the algae growing remains on the bottom of the tube. Therefore, a reduced amount of algae got into the cuvette and led to the reduction in fluorescence and Fv/Fm measurements seen. Despite this decline, the stress levels are similar between treatments and suggest that there is nothing in the growing environment that is harmful to *T. pseudonana,* supporting our hypothesis.

Nannochloropsis showed similar stress levels between treatments as well. Therefore, there is no obvious effect of treatment on the stress of the cells growing. This

result also supports the hypothesis that the concrete treatments would not have any effect on the growth and stress of algal strains.

Conclusions

I predicted that the different treatments (special concrete, normal concrete, and natural oyster shell) would not have any effect on water quality as measured by pH, conductivity, alkalinity, and calcium. This prediction is supported by these data. Although the pH and alkalinity in the aquaria with the special and normal concrete treatments was significantly lower than the aquaria with the natural shell and control treatments, this decrease is not biologically significant. The pH and alkalinity values are still within the range of normal seawater and well above the values that would significantly reduce calcification levels. Along with this result, conductivity and calcium levels were not significantly different between treatments. This suggests that the artificial treatments were not off-putting any additional ions into the surrounding environment. Finally, the calcium levels between treatments were also not significantly different. These data also suggest that the artificial substrates are not releasing larger quantities of calcium compared to the natural shell treatment.

I also predicted that the different treatments would not affect the growth or stress levels of biologically relevant algal strains known to be present in the Chesapeake Bay. This prediction was generally supported by the data. There were some strains that showed reduced algal growth in the presence of the special concrete treatment. However, this was only slightly lower growth than the normal concrete and natural shell treatments.

The stress levels of the algae growing showed a similar pattern with similar stress levels between treatments, when measurements could be taken.

The methods used here have been used in other studies (Liu and Huang 2000, Parkhill et al. 2001, Azim et al. 2002, Moss and Moss 2004, Ware and Thomson 2005, Wood et al. 2005, Arnold et al. 2006, Stepien and Klobus 2006, Scheitzer et al. 2013). However, the application of these methods to address the effects of artificial substrates for oyster restoration on localized water quality and algal growth has not been done. One related study examined the community development on artificial coral reef substrates over 30 months (McVey 1970). Concrete pipes were placed in the water and the algae that grew was identified and tracked throughout the study period (McVey 1970). Although algae growth was not the main purpose of the study, it was found that there was consistent algal growth throughout the study period suggesting that concrete does not negatively affect growth (McVey 1970). The results presented here are in line with what McVey found with concrete not showing any detrimental effects to algae growth. However, the lack of research on the larger community effects of artificial substrates exposes a gap in current knowledge. Further research should be conducted to ensure that no harmful effects come from the addition of exotic substrates into the marine system.

Overall, the hypothesis was supported by these data. It appears that the concrete artificial substrates do not cause immediate or substantial harm to the surrounding environment as measured by water quality and algal growth suggesting that there would not be any harmful effects on bottom-up trophic cascades. Together with previous data suggesting no initial adverse effects on oyster larval recruitment, I can conclude that

concrete artificial substrates (with and without augmented levels of calcium) can be used for oyster restoration efforts in the Chesapeake Bay.

IV. Conclusions and Implications

Conclusions

Settling Preference and Abundance

Over 14 weeks, spat preferred to settle on the natural shell treatment rather than the special and normal concrete treatments, potentially because the natural oyster shells provided a more ideal habitat. However, the abundance of settled spat on the special and normal concrete treatments was not statistically significantly different from each other suggesting there are no initial adverse effects of augmented calcium in the special concrete treatment.

Change in Abundance

Although there was more settled spat, the natural shell treatment ultimately lost more than twice as many spat than the special and normal concrete treatments. The greater initial settlement but eventual greater decline of spat over time could be explained by the theory of Ideal Free Distribution. The natural shell may have had the most resources and was the "ideal" habitat, but as more spat settled, their overall fitness decreased and individuals were lost. Additionally, there was greater competition for space to grow on the natural shells resulting, again, in individuals being lost. Finally, with greater settlement, there were higher densities of spat on the natural shells compared

to the concrete treatments potentially leading to greater predation of spat growing on natural shells resulting in lower numbers as the study went on.

Water Quality

There were no biologically significant effects of the special and normal concrete treatments on water quality. Both the pH and alkalinity values that were found to be significantly different from the natural shell treatments were still within normal pH and alkalinity levels. Conductivity and calcium levels were not significantly different between treatments. These data suggest that both concrete treatments show no adverse effects on water quality. Long-term studies should be conducted to determine how fast these substrates would dissolve and how that would effect water quality as well.

Algal Growth

There were no obvious effects of the special or normal concrete on the growth and stress levels of the 5 algal strains chosen for this study. Additional studies should be completed to examine a wider range of algal strains to ensure these results are consistent. However, these initial data suggest that these substrates are not off-putting harmful elements to the surrounding water that could harm bottom-up trophic cascades around oyster reef systems.

Implications

The precautionary principle of "do no harm" must be at the forefront with any restoration effort, especially in the Chesapeake Bay. Each year since 1998, the

Chesapeake Bay Foundation issues a State of the Bay report which takes into account three factors: (1) pollution, (2) habitat, and (3) fisheries. For each category, the Bay is given a score out of 100 and then all three are averaged to give the Bay an overall "health index". In 1998, the State of the Bay gave the Chesapeake Bay a 27. In 2016, the score had only risen to 34 despite numerous policy regulations. With restoration in the Chesapeake Bay, the goal is to improve the quality and health of the water and the organisms living there. Further work needs to be done, but it is imperative that researchers are taking into consideration the larger implications of restoration efforts.

The goal of this study was to piece together the effects of using artificial substrates with augmented levels of calcium for oyster restoration. This was done by exploring how this artificial substrate affected wild larval settlement, localized water quality in a laboratory setting, and the localized algal growth of 5 relevant algae species. By combining and studying several ecosystem factors, we can better inform how this artificial substrate would affect the overall food web and organismal interactions at potential restoration sites in the Chesapeake Bay.

We found that the artificial substrate with augmented levels of calcium had statistically similar larval settlement suggesting no initial adverse effects on recruitment. In addition, we found that this substrate showed no adverse effects on water quality or on algal growth. Therefore, we can conclude that the use of an artificial substrate with augmented levels of calcium to better mimic natural oyster shells can be used as a restoration substrate in the Chesapeake Bay to help reestablish oyster populations.

To better support this conclusion, an initial comparison on the ease of use for each substrate and its success of oyster larval recruitment (Table 4.1) was developed.

Natural shells are the more successful substrate when it comes to larval recruitment, but are difficult to acquire and use because oyster populations are so low. Both concrete substrates have lower recruitment success compared to natural oyster shells. However, they are relatively inexpensive to use and easy to make. A score between 0 and 1 was given for the ease of use of each substrate with lower scores signifying less ease of use. The low ease of use of natural oysters shells resulted in a lower score (0.25) compared to both concrete types, which are easy to use and inexpensive (0.75; Table 4.1). The same scoring system (0-1) was used to score the substrates for oyster recruitment success with higher scores signifying greater success. Natural shells received a score of 1, since they are the preferred substrate, and both concrete types received a score of 0.5 since they had statistically similar recruitment success compared to each other, but less success than the natural shells (Table 4.1). The final ratios were compared and higher numbers signified a better-suited substrate for restoration efforts. These ratios resulted in the natural shells being less beneficial (0.25) to restoration efforts than both of the concrete types (1.5).

Table 4.1. Comparing Substrates The ease of use and recruitment success was determined for each substrate. Low ease of use resulted in a lower score. High recruitment success resulted in a higher score. The computed ratio was evaluated by dividing the ease of use value by the recruitment success value.

Substrate	Ease of Use	Recruitment Success	Computed Ratio
Special Concrete	0.75	0.5	1.5
Normal Concrete	0.75	0.5	1.5
Natural Shell	0.25	1	0.25

With all pieces of evidence combined, this study reveals that concrete artificial substrates are beneficial as restoration substrates for oyster reefs. In addition, there are

no immediate adverse effects of using a concrete substrate with augmented levels of calcium to better mimic natural oyster shells. Long-term studies do need to be conducted, however, to determine the lasting effects of these substrates as oyster restoration tools in the Chesapeake Bay.

Appendix I

Table A1 Spat Counts. Number of spat in each experimental bag throughout the study period. Each bag contained 20 shell units and there were four bags for each shell type. Totals are for each shell type on each date of data collection. SC = special concrete, NC = normal concrete, NS = natural shell.

Bag	Spat on 7/27/2016	Spat on 8/12/2016	Spat on 8/24/2016	Spat on 9/11/2016	Spat on 9/24/2016	Spat on 10/9/2016	Spat on 10/23/2017
SC1	9	26	37	35	34	33	31
SC2	8	16	33	33	31	32	27
SC3	9	37	53	47	48	47	41
SC4	10	59	69	61	58	56	50
Total	**36**	**138**	**192**	**176**	**171**	**168**	**149**
NC1	12	44	49	44	41	38	32
NC2	9	33	60	52	49	46	44
NC3	15	60	67	61	57	57	49
NC4	4	24	53	54	56	52	49
Total	**40**	**161**	**229**	**211**	**203**	**193**	**174**
NS1	14	63	92	91	77	73	69
NS2	16	67	109	107	102	100	98
NS3	21	116	128	104	92	92	78
NS4	29	79	165	152	148	136	120
Total	**80**	**325**	**494**	**454**	**419**	**401**	**365**

Table A2 Pitman Cove Water Quality. Water quality measurements taken throughout the study period. Measurements taken by the associated Vernier probes.

Date	Time	Temperature	Salinity	pH
7/11/16	11:53am	30.50	15	8.22
7/27/16	2:42pm	34.5	16.7	8.32
8/12/16	2:50pm	32.4	14.8	8.25
8/24/16	1:05pm	30.3	16.5	8.3
9/11/16	1:21pm	28.2	12.5	8.09
9/24/16	1:40pm	26.5	9.7	8.05
10/9/16	3:29pm	21.2	10.2	8.1
10/23/16	3:12pm	21	10.4	8.03

Table A3 Average Water Quality Measurements per Treatment. Water quality measurements used for analysis. There were three tanks per treatment type. The average pH, conductivity, alkalinity, and calcium for each tank for the entire study period are shown.

Tank	pH	Conductivity	Alkalinity	Calcium
SC1	8.22	4432.5	137.2	498.1
SC2	8.24	4426.2	123.2	497.8
SC3	8.35	4411.1	139.3	466.6
NC1	8.22	4439.8	134.7	512.3
NC2	8.37	4447.3	130.2	454.3
NC3	8.37	4473.7	127.7	466.3
NS1	8.38	4428.5	200.7	502.2
NS2	8.45	4427.8	177	484
NS3	8.51	4424.6	211.2	485.6
Control	8.51	4414.6	213.6	492.7

Table A4 Instant Ocean Additions. Instant Ocean was added to each aquarium throughout the study period to ensure the salinity remained between 10 and 15 ppt.

Date	Instant Ocean Added (g)
8/30/16	600
9/10/16	100
9/18/16	250
9/30/16	300
10/10/16	200
10/22/16	250
11/3/16	300
11/15/16	200

Appendix II

II. Larval Settlement on Varying Substrates – R code

```
#---------------------------------Initial importing of data-------------------------------
file.choose(new=TRUE)      #import .csv of settlement data on each unit at each date
spat=read.csv([filename])
spat

#------------------------Converting the Shell ID's to factors rather than numbers-----------
spat$Shell.ID=factor(spat$Shell.ID)       #transforming the Shell.ID numbers to factors
spat$Bag=factor(spat$Bag)                 #transforming the Bag numbers to factors

##ordering the date factors
spat$Date=factor(spat$Date, levels=c("11-Jul", "27-Jul", "12-Aug", "24-Aug", "11-Sep",
"24-Sep", "9-Oct", "23-Oct"))

str(spat)   #looking at the data

#-------------------------------------Initial Analysis----------------------------------
mean(spat$Spat[spat$Type=="SC"])
mean(spat$Spat[spat$Type=="NC"])
mean(spat$Spat[spat$Type=="NS"])

median(spat$Spat[spat$Type=="SC"])
median(spat$Spat[spat$Type=="NC"])
median(spat$Spat[spat$Type=="NS"])

#--------------------------------Frequency of spat landing on each shell type------------------
par(mfrow=c(1, 3))

hist(spat$Spat[spat$Type=="SC"], main="Frequency of Spat Landing on SC", ylab =
"Frequency", xlab = "Number of Spat",
    ylim=c(0, 500))
hist(spat$Spat[spat$Type=="NC"], main="Frequency of Spat Landing on NC", ylab =
"Frequency", xlab = "Number of Spat",
    ylim=c(0, 500))
hist(spat$Spat[spat$Type=="NS"], main="Frequency of Spat Landing on NS", ylab =
"Frequency", xlab = "Number of Spat",
    ylim=c(0, 500))

par(mfrow=c(1,1))

#----------------------SHAPIRO-WILK for EACH SHELL TYPE to determine normality---
shapiro.test(spat$Spat[spat$Type=="SC"])
summary(spat$Spat[spat$Type=="SC"])
```

```
IQR(spat$Spat[spat$Type=="SC"])
sd(spat$Spat[spat$Type=="SC"])
boxplot(spat$Spat[spat$Type=="SC"], main="Spat on SC")
shapiro.test(spat$Spat[spat$Type=="NC"])
summary(spat$Spat[spat$Type=="NC"])
IQR(spat$Spat[spat$Type=="NC"])
sd(spat$Spat[spat$Type=="NC"])
boxplot(spat$Spat[spat$Type=="NC"], main="Spat on NC")

shapiro.test(spat$Spat[spat$Type=="NS"])
summary(spat$Spat[spat$Type=="NS"])
IQR(spat$Spat[spat$Type=="NS"])
sd(spat$Spat[spat$Type=="NS"])
boxplot(spat$Spat[spat$Type=="NS"], main="Spat on NS")

#----------------------SHAPIRO-WILK for EACH BAG to determine normality--------
shapiro.test(spat$Spat[spat$Bag.ID=="SC1"])
shapiro.test(spat$Spat[spat$Bag.ID=="SC2"])
shapiro.test(spat$Spat[spat$Bag.ID=="SC3"])
shapiro.test(spat$Spat[spat$Bag.ID=="SC4"])

shapiro.test(spat$Spat[spat$Bag.ID=="NC1"])
shapiro.test(spat$Spat[spat$Bag.ID=="NC2"])
shapiro.test(spat$Spat[spat$Bag.ID=="NC3"])
shapiro.test(spat$Spat[spat$Bag.ID=="NC4"])

shapiro.test(spat$Spat[spat$Bag.ID=="NS1"])
shapiro.test(spat$Spat[spat$Bag.ID=="NS2"])
shapiro.test(spat$Spat[spat$Bag.ID=="NS3"])
shapiro.test(spat$Spat[spat$Bag.ID=="NS4"])

#---------------------------------------plot spat # over time----------------------------------------
plot(x=spat$Date, y=spat$Spat, xlab= "Time", ylab="Number of Spat", main = " Total
Recruitment Over Time")

#-----------------------------boxplot spat # per shell type on all collection days---------------
spat$Date=factor(spat$Date, levels=c("11-Jul", "27-Jul", "12-Aug", "24-Aug", "11-Sep",
"24-Sep", "9-Oct", "23-Oct"))

spat$Type = factor(spat$Type, levels=c("SC", "NC", "NS"))

par(mfrow=c(4, 2))
par(mar=c(4,5.3,1.2,1.2))
plot(x=spat$Type[spat$Date=="27-Jul"], y=spat$Spat[spat$Date=="27-Jul"], ylab="",
xlab="",
    las=1,cex.axis = 1.8, col=c("gray32", "gray66", "gray91"), ylim=c(0, 20))
```

```
mtext("Spat Present (#)", side = 2, line = 3.2, cex = 1.5, font=2)
legend(locator(1), legend=c("July 27"), bty="n", cex=1.8)
par(mar=c(4,5.3,1.2,1.2))
plot(x=spat$Type[spat$Date=="12-Aug"], y=spat$Spat[spat$Date=="12-Aug"], ylab="",
xlab="",
    las=1,cex.axis = 1.8, col=c("gray32", "gray66", "gray91"), ylim=c(0, 20))
legend(locator(1), legend=c("August 12"), bty="n", cex=1.8)
par(mar=c(4,5.3,1.2,1.2))
plot(x=spat$Type[spat$Date=="24-Aug"], y=spat$Spat[spat$Date=="24-Aug"], ylab="",
xlab="",
    las=1,cex.axis = 1.8, col=c("gray32", "gray66", "gray91"), ylim=c(0, 20))
mtext("Spat Present (#)", side = 2, line = 3.2, cex = 1.5, font=2)
legend(locator(1), legend=c("August 24"), bty="n", cex=1.8)

par(mar=c(4,5.3,1.2,1.2))
plot(x=spat$Type[spat$Date=="11-Sep"], y=spat$Spat[spat$Date=="11-Sep"], ylab="",
xlab="",
    las=1,cex.axis = 1.8, col=c("gray32", "gray66", "gray91"), ylim=c(0, 20))
legend(locator(1), legend=c("September 11"), bty="n", cex=1.8)

par(mar=c(4,5.3,1.2,1.2))
plot(x=spat$Type[spat$Date=="24-Sep"], y=spat$Spat[spat$Date=="24-Sep"], ylab="",
xlab="",
    las=1,cex.axis = 1.8, col=c("gray32", "gray66", "gray91"), ylim=c(0, 20))
mtext("Spat Present (#)", side = 2, line = 3.2, cex = 1.5, font=2)
legend(locator(1), legend=c("September 24"), bty="n", cex=1.8)

par(mar=c(4,5.3,1.2,1.2))
plot(x=spat$Type[spat$Date=="9-Oct"], y=spat$Spat[spat$Date=="9-Oct"], ylab="",
xlab="",
    las=1,cex.axis = 1.8, col=c("gray32", "gray66", "gray91"), ylim=c(0, 20))
mtext("Shell Type", side = 1, line = 4, cex = 1.5, font=2)
legend(locator(1), legend=c("October 9"), bty="n", cex=1.8)

par(mar=c(5.3,5.3,1.2,1.2))
plot(x=spat$Type[spat$Date=="23-Oct"], y=spat$Spat[spat$Date=="23-Oct"], ylab="",
xlab="",
    las=1,cex.axis = 1.8, col=c("gray32", "gray66", "gray91"), ylim=c(0, 20))
mtext("Shell Type", side = 1, line = 3.8, cex = 1.5, font=2)
mtext("Spat Present (#)", side = 2, line = 3.2, cex = 1.5, font=2)
legend(locator(1), legend=c("October 23"), bty="n", cex=1.8)

sort(spat$Spat[spat$Type == "SC"], decreasing=FALSE)
sort(spat$Spat[spat$Type == "NC"], decreasing=FALSE)
sort(spat$Spat[spat$Type == "NS"], decreasing=FALSE)
```

```
summary(spat$Spat[spat$Type == "SC"])
summary(spat$Spat[spat$Type == "NC"])
summary(spat$Spat[spat$Type == "NS"])
#----------------------plot spat # per shell type per date-------------------------------
par(mfrow=c(1, 3))

plot(y=spat$Spat[spat$Type=="SC"], x=spat$Date[spat$Type=="SC"], ylab="Number of
Spat", xlab="Date",
    main="Number of Spat on SC Over Time", ylim=c(0, max(spat$Spat)))

plot(y=spat$Spat[spat$Type=="NC"], x=spat$Date[spat$Type=="NC"], ylab="Number
of Spat", xlab="Date",
    main="Number of Spat on NC Over Time", ylim=c(0, max(spat$Spat)))

plot(y=spat$Spat[spat$Type=="NS"], x=spat$Date[spat$Type=="NS"], ylab="Number
of Spat", xlab="Date",
    main="Number of Spat on NS Over Time", ylim=c(0, max(spat$Spat)))

par(mfrow=c(1,1))

#--------------------------------------STATISTICAL TESTS ------------------------------
sum(spat$Spat[spat$Date=="23-Oct" & spat$Type=="SC"])
sum(spat$Spat[spat$Date=="23-Oct" & spat$Type=="NC"])
sum(spat$Spat[spat$Date=="23-Oct" & spat$Type=="NS"])

summary(spat$Spat[spat$Date=="23-Oct" & spat$Type=="SC"])
summary(spat$Spat[spat$Date=="23-Oct" & spat$Type=="NC"])
summary(spat$Spat[spat$Date=="23-Oct" & spat$Type=="NS"])

median(spat$Spat[spat$Type=="SC"])
median(spat$Spat[spat$Type=="NC"])
median(spat$Spat[spat$Type=="NS"])

sd(spat$Spat[spat$Type=="SC"])
sd(spat$Spat[spat$Type=="NC"])
sd(spat$Spat[spat$Type=="NS"])

var(spat$Spat[spat$Type=="SC"])
var(spat$Spat[spat$Type=="NC"])
var(spat$Spat[spat$Type=="NS"])

#-----------------------KRUSKAL WALLIS (AT EACH TIME)-----------------------------
library(PMCMR)

#----------July 27----------
```

```
hist(spat$Spat[spat$Type=="SC" & spat$Date=="27-Jul"], xlab="Spat", main="Spat on
SC-July 27", xlim=c(0, 2),   #looking at the distribution
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="SC" & spat$Date=="27-Jul"])
hist(spat$Spat[spat$Type=="NC" & spat$Date=="27-Jul"], xlab="Spat", main="Spat on
NC-July 27",xlim=c(0, 2),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="NC" & spat$Date=="27-Jul"])
hist(spat$Spat[spat$Type=="NS" & spat$Date=="27-Jul"], xlab="Spat", main="Spat on
NS-July 27", xlim=c(0, 4),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="NS" & spat$Date=="27-Jul"])       #testing for
normality
kruskal.test(spat$Spat[241:480]~(spat$Type[spat$Date=="27-Jul"]))     #testing for
significance
posthoc.kruskal.nemenyi.test(spat$Spat[241:480]~(spat$Type[spat$Date=="27-Jul"]))
#post hoc to determine differences

Jul_27 <- spat[240:480, ]    #separating just the July 27 collection date
Jul_27
install.packages("coin")
library(coin)
wilcox_test(Spat ~ factor(Type),
data=Jul_27[Jul_27$Type=="NS"|Jul_27$Type=="SC",], distribution="exact")    #effect
size calculation
2.8862/sqrt(480)   #using the |Z value| divided by the total sample size (240 NS + 240
SC) to get the effect size
wilcox_test(Spat ~ factor(Type),
data=Jul_27[Jul_27$Type=="NS"|Jul_27$Type=="NC",], distribution="exact")    #effect
size calculation
-2.3894/sqrt(480)   #using the |Z value| divided by the total sample size (240 NS + 240
NC) to get the effect size

#----------August 12----------
hist(spat$Spat[spat$Type=="SC" & spat$Date=="12-Aug"], xlab="Spat", main="Spat on
SC-Aug 12", xlim=c(0, 12),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="SC" & spat$Date=="12-Aug"])
hist(spat$Spat[spat$Type=="NC" & spat$Date=="12-Aug"], xlab="Spat", main="Spat on
NC-Aug 12",xlim=c(0, 12),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="NC" & spat$Date=="12-Aug"])
hist(spat$Spat[spat$Type=="NS" & spat$Date=="12-Aug"], xlab="Spat", main="Spat on
NS-Aug 12", xlim=c(0, 20),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="NS" & spat$Date=="12-Aug"])
```

```
kruskal.test(spat$Spat[481:720]~(spat$Type[spat$Date=="12-Aug"]))
posthoc.kruskal.nemenyi.test(spat$Spat[481:720]~(spat$Type[spat$Date=="12-Aug"]))


Aug_12 <- spat[481:720, ]
Aug_12
wilcox_test(Spat ~ factor(Type),
data=Aug_12[Aug_12$Type=="NS"|Aug_12$Type=="SC",], distribution="exact")
5.5067/sqrt(480)
wilcox_test(Spat ~ factor(Type),
data=Aug_12[Aug_12$Type=="NS"|Aug_12$Type=="NC",], distribution="exact")
-4.6407/sqrt(480)

#----------August 24----------
hist(spat$Spat[spat$Type=="SC" & spat$Date=="24-Aug"], xlab="Spat", main="Spat on
SC-Aug 24", xlim=c(0, 20),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="SC" & spat$Date=="24-Aug"])
hist(spat$Spat[spat$Type=="NC" & spat$Date=="24-Aug"], xlab="Spat", main="Spat on
NC-Aug 24",xlim=c(0, 20),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="NC" & spat$Date=="24-Aug"])
hist(spat$Spat[spat$Type=="NS" & spat$Date=="24-Aug"], xlab="Spat", main="Spat on
NS-Aug 24", xlim=c(0, 20),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="NS" & spat$Date=="24-Aug"])
kruskal.test(spat$Spat[721:960]~(spat$Type[spat$Date=="24-Aug"]))
posthoc.kruskal.nemenyi.test(spat$Spat[721:960]~(spat$Type[spat$Date=="24-Aug"]))

Aug_24 <- spat[721:960, ]
Aug_24
wilcox_test(Spat ~ factor(Type),
data=Aug_24[Aug_24$Type=="NS"|Aug_24$Type=="SC",], distribution="exact")
6.5653/sqrt(480)
wilcox_test(Spat ~ factor(Type),
data=Aug_24[Aug_24$Type=="NS"|Aug_24$Type=="NC",], distribution="exact")
-5.8598/sqrt(480)

#----------September 11----------
hist(spat$Spat[spat$Type=="SC" & spat$Date=="11-Sep"], xlab="Spat", main="Spat on
SC-Sept 11", xlim=c(0, 20),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="SC" & spat$Date=="11-Sep"])
hist(spat$Spat[spat$Type=="NC" & spat$Date=="11-Sep"], xlab="Spat", main="Spat on
NC-Sept 11",xlim=c(0, 20),
    ylim=c(0, 80))
```

```
shapiro.test(spat$Spat[spat$Type=="NC" & spat$Date=="11-Sep"])
hist(spat$Spat[spat$Type=="NS" & spat$Date=="11-Sep"], xlab="Spat", main="Spat on
NS-Sept 11", xlim=c(0, 20),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="NS" & spat$Date=="11-Sep"])
kruskal.test(spat$Spat[961:1200]~(spat$Type[spat$Date=="11-Sep"]))
posthoc.kruskal.nemenyi.test(spat$Spat[961:1200]~(spat$Type[spat$Date=="11-Sep"]))

Sept_11 <- spat[961:1200, ]
Sept_11
wilcox_test(Spat ~ factor(Type),
data=Sept_11[Sept_11$Type=="NS"|Sept_11$Type=="SC",], distribution="exact")
6.1296/sqrt(480)
wilcox_test(Spat ~ factor(Type),
data=Sept_11[Sept_11$Type=="NS"|Sept_11$Type=="NC",], distribution="exact")
-5.5071/sqrt(480)

#----------September 24----------
hist(spat$Spat[spat$Type=="SC" & spat$Date=="24-Sep"], xlab="Spat", main="Spat on
SC-Sept 24", xlim=c(0, 20),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="SC" & spat$Date=="24-Sep"])
hist(spat$Spat[spat$Type=="NC" & spat$Date=="24-Sep"], xlab="Spat", main="Spat on
NC-Sept 24",xlim=c(0, 20),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="NC" & spat$Date=="24-Sep"])
hist(spat$Spat[spat$Type=="NS" & spat$Date=="24-Sep"], xlab="Spat", main="Spat on
NS-Sept 24", xlim=c(0, 20),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="NS" & spat$Date=="24-Sep"])
kruskal.test(spat$Spat[1201:1440]~(spat$Type[spat$Date=="24-Sep"]))
posthoc.kruskal.nemenyi.test(spat$Spat[1201:1440]~(spat$Type[spat$Date=="24-Sep"]))

Sept_24 <- spat[1201:1440, ]
Sept_24
wilcox_test(Spat ~ factor(Type),
data=Sept_24[Sept_24$Type=="NS"|Sept_24$Type=="SC",], distribution="exact")
5.9105/sqrt(480)
wilcox_test(Spat ~ factor(Type),
data=Sept_24[Sept_24$Type=="NS"|Sept_24$Type=="NC",], distribution="exact")
-5.2677/sqrt(480)

#----------October 9----------
hist(spat$Spat[spat$Type=="SC" & spat$Date=="9-Oct"], xlab="Spat", main="Spat on
SC-Oct 9", xlim=c(0, 20),
    ylim=c(0, 80))
```

```
shapiro.test(spat$Spat[spat$Type=="SC" & spat$Date=="9-Oct"])
hist(spat$Spat[spat$Type=="NC" & spat$Date=="9-Oct"], xlab="Spat", main="Spat on
NC-Oct 9",xlim=c(0, 20),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="NC" & spat$Date=="9-Oct"])
hist(spat$Spat[spat$Type=="NS" & spat$Date=="9-Oct"], xlab="Spat", main="Spat on
NS-Oct 9", xlim=c(0, 20),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="NS" & spat$Date=="9-Oct"])
kruskal.test(spat$Spat[1441:1680]~(spat$Type[spat$Date=="9-Oct"]))
posthoc.kruskal.nemenyi.test(spat$Spat[1441:1680]~(spat$Type[spat$Date=="9-Oct"]))

Oct_9 <- spat[1401:1680, ]
Oct_9
wilcox_test(Spat ~ factor(Type), data=Oct_9[Oct_9$Type=="NS"|Oct_9$Type=="SC",],
distribution="exact")
7.0113/sqrt(480)
wilcox_test(Spat ~ factor(Type), data=Oct_9[Oct_9$Type=="NS"|Oct_9$Type=="NC",],
distribution="exact")
-6.4762/sqrt(480)

#----------October 23----------
hist(spat$Spat[spat$Type=="SC" & spat$Date=="23-Oct"], xlab="Spat", main="Spat on
SC-Oct 23", xlim=c(0, 12),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="SC" & spat$Date=="23-Oct"])
hist(spat$Spat[spat$Type=="NC" & spat$Date=="23-Oct"], xlab="Spat", main="Spat on
NC-Oct 23",xlim=c(0, 20),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="NC" & spat$Date=="23-Oct"])
hist(spat$Spat[spat$Type=="NS" & spat$Date=="23-Oct"], xlab="Spat", main="Spat on
NS-Oct 23", xlim=c(0, 20),
    ylim=c(0, 80))
shapiro.test(spat$Spat[spat$Type=="NS" & spat$Date=="23-Oct"])
kruskal.test(spat$Spat[1681:1920]~(spat$Type[spat$Date=="23-Oct"]))
posthoc.kruskal.nemenyi.test(spat$Spat[1681:1920]~(spat$Type[spat$Date=="23-Oct"]))

Oct_23 <- spat[1681:1920, ]
Oct_23
wilcox_test(Spat ~ factor(Type),
data=Oct_23[Oct_23$Type=="NS"|Oct_23$Type=="SC",], distribution="exact")
6.0743/sqrt(480)
wilcox_test(Spat ~ factor(Type),
data=Oct_23[Oct_23$Type=="NS"|Oct_23$Type=="NC",], distribution="exact")
-5.3684/sqrt(480)
```

```
#------------------------CHI SQUARE AND POST HOC--------------------
install.packages("fifer")
library(fifer)

matrix_sums=matrix(c(sum(spat$Spat[spat$Date=="27-Jul" & spat$Type=="SC"]),
                sum(spat$Spat[spat$Date=="27-Jul" & spat$Type=="NC"]),
                sum(spat$Spat[spat$Date=="27-Jul" & spat$Type=="NS"]),
                sum(spat$Spat[spat$Date=="12-Aug" & spat$Type=="SC"]),
                sum(spat$Spat[spat$Date=="12-Aug" & spat$Type=="NC"]),
                sum(spat$Spat[spat$Date=="12-Aug" & spat$Type=="NS"]),
                sum(spat$Spat[spat$Date=="24-Aug" & spat$Type=="SC"]),
                sum(spat$Spat[spat$Date=="24-Aug" & spat$Type=="NC"]),
                sum(spat$Spat[spat$Date=="24-Aug" & spat$Type=="NS"]),
                sum(spat$Spat[spat$Date=="11-Sep" & spat$Type=="SC"]),
                sum(spat$Spat[spat$Date=="11-Sep" & spat$Type=="NC"]),
                sum(spat$Spat[spat$Date=="11-Sep" & spat$Type=="NS"]),
                sum(spat$Spat[spat$Date=="24-Sep" & spat$Type=="SC"]),
                sum(spat$Spat[spat$Date=="24-Sep" & spat$Type=="NC"]),
                sum(spat$Spat[spat$Date=="24-Sep" & spat$Type=="NS"]),
                sum(spat$Spat[spat$Date=="9-Oct" & spat$Type=="SC"]),
                sum(spat$Spat[spat$Date=="9-Oct" & spat$Type=="NC"]),
                sum(spat$Spat[spat$Date=="9-Oct" & spat$Type=="NS"]),
                sum(spat$Spat[spat$Date=="23-Oct" & spat$Type=="SC"]),
                sum(spat$Spat[spat$Date=="23-Oct" & spat$Type=="NC"]),
                sum(spat$Spat[spat$Date=="23-Oct" & spat$Type=="NS"])), ncol=7,
byrow=FALSE)      #creates a matrix with the total spat on each shell type at each time
colnames(matrix_sums) <-c("Jul 27", "Aug 12", "Aug 24", " Sept 11", " Sept 24", " Oct
9", "Oct 23")
row.names(matrix_sums) <-c("SC", "NC", "NS")

sums <- as.table(matrix_sums)
sums
chisq.test(sums[ , 7])      #chi ssquare test for the final collection date

#-------------------total spat counts-------------------------
par(mfrow=c(1,1))
par(mar=c(6.5, 6.5, 1.5, 2.1))
barplot(matrix_sums, beside=TRUE, ylim=c(0, 550), xlab= "", ylab = "",         #creating a
bar plot that shows the settlement on each type at each time
       las=1, cex.axis = 2, cex.names=2)
mtext("Collection Date", side = 1, line = 4.5, cex = 2.2, font=2)
mtext("Spat Present (#)", side = 2, line = 4.5, cex = 2.2, font=2)
legend("topright", c("Special Concrete", "Normal Concrete", "Natural Shell"),
fill=c("gray32", "gray66", "gray91"),
      bty="n", cex = 2)
```

```
text(x=7.5, y=320, "**", pos=3, cex=2)         #putting in the significance levels as
calculated from the kruskal wallis tests above
text(x=11.5, y=490, "**", pos=3, cex=2)
text(x=15.5, y=450, "**", pos=3, cex=2)
text(x=19.5, y=415, "**", pos=3, cex=2)
text(x=23.5, y=395, "**", pos=3, cex=2)
text(x=27.5, y=360, "**", pos=3, cex=2)
segments(x0 = 1.5, x1 = 3.5, y0 = 100, y1 = 100, lwd = c(2))
segments(x0 = 1.5, y0 = 100, x1 = 1.5, y1 = 95, lwd=c(2))
segments(x0 = 3.5, y0 = 100, x1 = 3.5, y1 = 95, lwd=c(2))
text(x= 2.5, y = 95, "*", pos=3, cex=2)
abline(h=0)

#---------------------------------Percent difference for each substrate for the last 4
collections-----
file.choose(new=TRUE)
perc = read.csv([filename])   #import csv file of the percent increase/decrease in spat on
each shell type from one collection date to the next
perc

mean(perc$Percent[perc$Shell=="NS"])
mean(perc$Percent[perc$Shell=="NC"])
mean(perc$Percent[perc$Shell=="SC"])

hist(perc$Percent[perc$Shell=="NS"])             #look at distributions
hist(perc$Percent[perc$Shell=="NC"])
hist(perc$Percent[perc$Shell=="SC"])

shapiro.test(perc$Percent[perc$Shell=="NS"])      #test for normality
shapiro.test(perc$Percent[perc$Shell=="NC"])
shapiro.test(perc$Percent[perc$Shell=="SC"])

anova(aov(perc$Percent~perc$Shell))              #test for significance

#---------------------------------Percent change over time-----------------------------------
library(fifer)

matrix_percent=matrix(c(283.3, 302.5, 306.3,        #create a matrix with the percent +/-
from one date to the next
                        39.1, 42.2, 52,
                        -6.8, -7.9, -8.1,
                        -4.5, -3.8, -7.7,
                        -1.8, -5.9, -4.8,
                        -11.8, -8.9, -8.5), ncol=6, byrow=FALSE)
colnames(matrix_percent) = c("Jul 27-\nAug 12", "Aug 12-\nAug 24", "Aug 24-\nSept
11",
```

```
                    "Sept 11-\nSept 24", "Sept 24-\nOct 9", "Oct 9-\nOct 23")
row.names(matrix_percent) <-c("SC", "NC", "NS")
matrix_percent

par(mfrow=c(1,1))       #plot the percent +/-
par(mar=c(6.5, 6.5, 2.1, 2.1))
barplot(matrix_percent, beside=TRUE, ylim=c(-20, 300), xlab= "", ylab = "",
      las=1, cex.axis = 2, cex.names = 2)
abline(h=0)
mtext("Collection Date", side = 1, line = 4, cex = 2.2, font=2)
mtext("Increase/Decrease in Spat (%)", side = 2, line = 4.5, cex = 2.2, font=2)
legend("topright", c("Special Concrete", "Normal Concrete", "Natural Shell"),
fill=c("gray32", "gray66", "gray91"),
     bty="n", cex = 2)

par(mar=c(6.5, 7.5, 2.1, 2.1))
barplot(matrix_percent[, 3:6], beside=TRUE, ylim=c(-12, 0), xlab= "", ylab = "",
      las=1, cex.axis = 5, cex.names=0.01)
box()
```

III. Effect of Substrates on Water Quality and Algal Growth – R code

Water Quality

```
file.choose(new=TRUE)
chem=read.csv([filename])     #import csv file of all water quality measurements taken
                              througout the study period
chem

str(chem)
chem$Tank=factor(chem$Tank)
chem$Date=factor(chem$Date, levels=c("9/6/16", "9/8/16", "9/10/16", "9/12/16",
                                     "9/14/16", "9/16/16", "9/18/16",
                                     "9/20/16", "9/22/16", "9/24/16", "9/26/16",
                                     "9/28/16", "9/30/16", "10/2/16", "10/4/16",
                                     "10/6/16", "10/8/16", "10/10/16", "10/12/16",
                                     "10/14/16", "10/16/16", "10/18/16", "10/20/16",
                                     "10/22/16", "10/24/16", "10/26/16", "10/28/16",
                                     "10/30/16", "11/1/16", "11/3/16", "11/5/16",
                                     "11/7/16", "11/9/16", "11/11/16", "11/13/16",
                                     "11/15/16", "11/17/16", "11/19/16", "11/21/16",
                                    "11/23/16", "11/25/16", "11/27/16", "11/29/16"))
                                    #ordering the dates of data collection
str(chem)
```

```
treatment <- rep(c("SC", "NC", "SC", "NS", "NS", "NC", "SC", "NS", "NC", "C"), 43)
                    #adding a column with what was in each tank
chem <- data.frame(treatment, chem)
chem

chem_date = as.numeric(chem$Date, levels = c("9/6/16", "9/8/16", "9/10/16", "9/12/16",
                                             "9/14/16", "9/16/16", "9/18/16",
                                              "9/20/16", "9/22/16", "9/24/16", "9/26/16",
                                             "9/28/16", "9/30/16", "10/2/16", "10/4/16",
                                              "10/6/16", "10/8/16", "10/10/16", "10/12/16",
                                             "10/14/16", "10/16/16", "10/18/16", "10/20/16",
                                             "10/22/16", "10/24/16", "10/26/16", "10/28/16",
                                             "10/30/16", "11/1/16", "11/3/16", "11/5/16",
                                              "11/7/16", "11/9/16", "11/11/16", "11/13/16",
                                              "11/15/16", "11/17/16", "11/19/16",
                                              "11/21/16", "11/23/16", "11/25/16",
                                              "11/27/16", "11/29/16"))       #adding dates that
                                              are numeric for graphing
chem_date = data.frame(chem_date, chem)
#-----------------------------DETERMINING NORMALITY--------------------
#pH measurements
par(mfrow=c(5, 4))
for(i in c(1, 4:6, 9, 10, 13:16)) {
  hist(chem$pH[chem$Tank==i], main=expression(paste("Tank ", i)))
  qqnorm(chem$pH[chem$Tank==i]); qqline(chem$pH[chem$Tank==i])
  print(shapiro.test(chem$pH[chem$Tank==i]))
}

#conductivity
par(mfrow=c(5, 4))
for(i in c(1, 4:6, 9, 10, 13:16)) {
  hist(chem$Conductivity[chem$Tank==i], main=expression(paste("Tank ", i)))
  qqnorm(chem$Conductivity[chem$Tank==i]);
qqline(chem$Conductivity[chem$Tank==i])
  print(shapiro.test(chem$Conductivity[chem$Tank==i]))
}

#alkalinity
par(mfrow=c(5, 4))
for(i in c(1, 4:6, 9, 10, 13:16)) {
  hist(chem$Alkalinity[chem$Tank==i], main=expression(paste("Tank ", i)))
  qqnorm(chem$Alkalinity[chem$Tank==i]); qqline(chem$Alkalinity[chem$Tank==i])
  print(shapiro.test(chem$Alkalinity[chem$Tank==i]))
}

#calcium
```

```
par(mfrow=c(5, 4))
for(i in c(1, 4:6, 9, 10, 13:16)) {
  hist(chem$Calcium[chem$Tank==i], main=expression(paste("Tank ", i)))
  qqnorm(chem$Calcium[chem$Tank==i]); qqline(chem$Calcium[chem$Tank==i])
  print(shapiro.test(chem$Calcium[chem$Tank==i]))
}

#------------------------------MEAN/MEDIAN STATISTICS-----------------------------------
#pH
for(i in c(1, 4:6, 9, 10, 13:16)) {
  print(mean(chem$pH[chem$Tank==i]))
  print(median(chem$pH[chem$Tank==i]))}

#Temp
for(i in c(1, 4:6, 9, 10, 13:16)) {
  print(mean(chem$Temp[chem$Tank==i]))
  print(median(chem$Temp[chem$Tank==i]))}

#Salinity
for(i in c(1, 4:6, 9, 10, 13:16)) {
  print(mean(chem$Salinity[chem$Tank==i], na.rm=TRUE))
  print(median(chem$Salinity[chem$Tank==i], na.rm=TRUE))}

#Conductivity
for(i in c(1, 4:6, 9, 10, 13:16)) {
  print(mean(chem$Conductivity[chem$Tank==i]))
  print(median(chem$Conductivity[chem$Tank==i]))}

#Alkalinity
for(i in c(1, 4:6, 9, 10, 13:16)) {
  print(mean(chem$Alkalinity[chem$Tank==i], na.rm = TRUE))
  print(median(chem$Alkalinity[chem$Tank==i], na.rm = TRUE))}

#Calcium
for(i in c(1, 4:6, 9, 10, 13:16)) {
  print(mean(chem$Calcium[chem$Tank==i], na.rm = TRUE))
  print(median(chem$Calcium[chem$Tank==i], na.rm = TRUE))}

#-----------------COMPARING SAME CHEMISTRY ACROSS TREATMENTS-----------
#-------pH--------
par(mfrow=c(1,1))
lev<-c("6","9","14","1","5","13","4","10","15","16")
i<-1
plot(y=chem_date$pH[chem_date$Tank==lev[i]],
    x=chem_date$Date[chem_date$Tank==lev[i]], ylim=c(8, 9),
    ylab=c("pH"), xlab=c("Date"), pch=8)
```

```
for(i in 2:10){
  points(y=chem_date$pH[chem_date$Tank==lev[i]],
        x=chem_date$Date[chem_date$Tank==lev[i]],pch=i)}
legend(locator(1),lev,pch=1:10)

#-----Temp---------
par(mfrow=c(1,1))
lev<-c("6","9","14","1","5","13","4","10","15","16")
i<-1
plot(y=chem_date$Temp[chem_date$Tank==lev[i]],
    x=chem_date$Date[chem_date$Tank==lev[i]], ylim=c(10, 30),
    ylab=c("Temp"), xlab=c("Date"), pch=8)
for(i in 2:10){
  points(y=chem_date$Temp[chem_date$Tank==lev[i]],
        x=chem_date$Date[chem_date$Tank==lev[i]],pch=i)}
legend(locator(1),lev,pch=1:10)

hist(chem_date$Temp)
shapiro.test(chem_date$Temp)
mean(chem_date$Temp)
sd(chem_date$Temp)
median(chem_end$Temp)

#--------Salinity---------
par(mfrow=c(1,1))
lev<-c("6","9","14","1","5","13","4","10","15","16")
i<-1
plot(y=chem_date$Salinity[chem_date$Tank==lev[i]],
    x=chem_date$Date[chem_date$Tank==lev[i]], ylim=c(0, 20),
    ylab=c("Salinity"), xlab=c("Date"), pch=8)
for(i in 2:10){
  points(y=chem_date$Salinity[chem_date$Tank==lev[i]],
        x=chem_date$Date[chem_date$Tank==lev[i]],pch=i)}
legend(locator(1),lev,pch=1:10)

hist(chem_date$Salinity)
mean(chem_date$Salinity, na.rm=TRUE)
sd(chem_date$Salinity, na.rm=TRUE)

#-----Conductivity----------
par(mfrow=c(1,1))
lev<-c("6","9","14","1","5","13","4","10","15","16")
i<-1
plot(y=chem_date$Conductivity[chem_date$Tank==lev[i]],
    x=chem_date$Date[chem_date$Tank==lev[i]], ylim=c(3000, 5000),
    ylab=c("Conductivity"), xlab=c("Date"), pch=8)
```

```
for(i in 2:10){
  points(y=chem_date$Conductivity[chem_date$Tank==lev[i]],
        x=chem_date$Date[chem_date$Tank==lev[i]],pch=i)}
legend(locator(1),lev,pch=1:10)

#--------Alkalinity--------
par(mfrow=c(1,1))
lev<-c("6","9","14","1","5","13","4","10","15","16")
i<-1
plot(y=chem_date$Alkalinity[chem_date$Tank==lev[i]],
    x=chem_date$Date[chem_date$Tank==lev[i]], ylim=c(50, 300),
    ylab=c("Alkalinity"), xlab=c("Date"), pch=8)
for(i in 2:10){
  points(y=chem_date$Alkalinity[chem_date$Tank==lev[i]],
        x=chem_date$Date[chem_date$Tank==lev[i]],pch=i)}
legend(locator(1),lev,pch=1:10)

#---------Calcium----------
par(mfrow=c(1,1))
lev<-c("6","9","14","1","5","13","4","10","15","16")
i<-1
plot(y=chem_date$Calcium[chem_date$Tank==lev[i]],
    x=chem_date$Date[chem_date$Tank==lev[i]], ylim=c(200, 600),
    ylab=c("Calcium"), xlab=c("Date"), pch=8)
for(i in 2:10){
  points(y=chem_date$Calcium[chem_date$Tank==lev[i]],
        x=chem_date$Date[chem_date$Tank==lev[i]],pch=i)}
legend(locator(1),lev,pch=1:10)

#---------------FINDING AVERAGES FOR EACH TANK FROM 10/4 ON----------------
file.choose(new=TRUE)
chem_end = read.csv([filename])          #import csv file with data just from 10/12 and on
chem_end

chem_end$Date = factor(chem_end$Date, levels=c("10/12/16", "10/18/16", "10/26/16",
                                               "11/1/16", "11/9/16", "11/15/16",
                                               "11/23/16", "11/29/16"))   #ordering
                                               the dates
treatment = c("SC", "NC", "SC", "NS", "NS", "NC", "SC", "NS", "NC", "C")      #adding
                                                                    a column with
                                                                    the treatments
                                                                    in each tank
chem_end = data.frame(treatment, chem_end)
chem_end

treatment2 = c("NS", "NS", "NS", "SC", "SC", "SC", "NC", "NC", "NC", "C")
```

```
#AVERAGE PH--> finding the average pH in each tank for the study period from 10/12
and on
T6ph = mean(chem_end$pH[chem_end$Tank=="6"])
T9ph = mean(chem_end$pH[chem_end$Tank=="9"])
T14ph = mean(chem_end$pH[chem_end$Tank=="14"])
T1ph = mean(chem_end$pH[chem_end$Tank=="1"])
T5ph = mean(chem_end$pH[chem_end$Tank=="5"])
T13ph = mean(chem_end$pH[chem_end$Tank=="13"])
T4ph = mean(chem_end$pH[chem_end$Tank=="4"])
T10ph = mean(chem_end$pH[chem_end$Tank=="10"])
T15ph = mean(chem_end$pH[chem_end$Tank=="15"])
T16ph = mean(chem_end$pH[chem_end$Tank=="16"])
phs <- c(T6ph, T9ph, T14ph, T1ph, T5ph, T13ph, T4ph, T10ph, T15ph, T16ph)
tanks <- c(6, 9, 14, 1, 5, 13, 4, 10, 15, 16)

avg_ph <- data.frame(tanks, treatment2, phs)
avg_ph

#AVERAGE CONDUCTIVITY--> finding the average conductivity in each tank for the
study period from 10/12 and on
T6cond = mean(chem_end$Conductivity[chem_end$Tank=="6"])
T9cond = mean(chem_end$Conductivity[chem_end$Tank=="9"])
T14cond = mean(chem_end$Conductivity[chem_end$Tank=="14"])
T1cond = mean(chem_end$Conductivity[chem_end$Tank=="1"])
T5cond = mean(chem_end$Conductivity[chem_end$Tank=="5"])
T13cond = mean(chem_end$Conductivity[chem_end$Tank=="13"])
T4cond = mean(chem_end$Conductivity[chem_end$Tank=="4"])
T10cond = mean(chem_end$Conductivity[chem_end$Tank=="10"])
T15cond = mean(chem_end$Conductivity[chem_end$Tank=="15"])
T16cond = mean(chem_end$Conductivity[chem_end$Tank=="16"])
cond <- c(T6cond, T9cond, T14cond, T1cond, T5cond, T13cond, T4cond, T10cond,
T15cond, T16cond)
tanks <- c(6, 9, 14, 1, 5, 13, 4, 10, 15, 16)

avg_cond <- data.frame(tanks, treatment2, cond)
avg_cond

#AVERAGE ALKALINITY--> finding the average alkalinity in each tank for the study
period from 10/12 and on
T6alk = mean(chem_end$Alkalinity[chem_end$Tank=="6"])
T9alk = mean(chem_end$Alkalinity[chem_end$Tank=="9"])
T14alk = mean(chem_end$Alkalinity[chem_end$Tank=="14"])
T1alk = mean(chem_end$Alkalinity[chem_end$Tank=="1"])
T5alk = mean(chem_end$Alkalinity[chem_end$Tank=="5"])
T13alk = mean(chem_end$Alkalinity[chem_end$Tank=="13"])
```

```
T4alk = mean(chem_end$Alkalinity[chem_end$Tank=="4"])
T10alk = mean(chem_end$Alkalinity[chem_end$Tank=="10"])
T15alk = mean(chem_end$Alkalinity[chem_end$Tank=="15"])
T16alk = mean(chem_end$Alkalinity[chem_end$Tank=="16"])
alk <- c(T6alk, T9alk, T14alk, T1alk, T5alk, T13alk, T4alk, T10alk, T15alk, T16alk)
tanks <- c(6, 9, 14, 1, 5, 13, 4, 10, 15, 16)

avg_alk <- data.frame(tanks, treatment2, alk)
avg_alk

#AVERAGE CALCIUM--> finding the average calcium in each tank for the study period
from 10/12 and on
T6ca = mean(chem_end$Calcium[chem_end$Tank=="6"])
T9ca = mean(chem_end$Calcium[chem_end$Tank=="9"])
T14ca = mean(chem_end$Calcium[chem_end$Tank=="14"])
T1ca = mean(chem_end$Calcium[chem_end$Tank=="1"])
T5ca = mean(chem_end$Calcium[chem_end$Tank=="5"])
T13ca = mean(chem_end$Calcium[chem_end$Tank=="13"])
T4ca = mean(chem_end$Calcium[chem_end$Tank=="4"])
T10ca = mean(chem_end$Calcium[chem_end$Tank=="10"])
T15ca = mean(chem_end$Calcium[chem_end$Tank=="15"])
T16ca = mean(chem_end$Calcium[chem_end$Tank=="16"])
ca <- c(T6ca, T9ca, T14ca, T1ca, T5ca, T13ca, T4ca, T10ca, T15ca, T16ca)
tanks <- c(6, 9, 14, 1, 5, 13, 4, 10, 15, 16)

avg_ca <- data.frame(tanks, treatment2, ca)
avg_ca

#ALL AVERAGES DATA FRAME-->putting all the averages into a data frame
all_avg <- data.frame(tanks, treatment2, phs, cond, alk, ca)
all_avg

cor(phs, alk)      #determine if there is any correlation between variables
cor(phs, cond)
cor(ca, phs)
cor(alk, cond)
cor(ca, cond)
cor(alk, ca)

#--DETERMINING NORMALITY FOR EACH CHEM WITHIN EACH TREATMENT
#PH NORMALITY
ph_treatment = c("NS", "SC", "NC", "C")
par(mfrow=c(4, 2))
for(i in ph_treatment) {
  hist(chem_end$pH[chem_end$treatment==i], main=c("pH of ",i))
  a = shapiro.test(chem_end$pH[chem_end$treatment==i])
```

```
  legend("topright", legend = a$p.value)
  qqnorm(chem_end$pH[chem_end$treatment==i])
  qqline(chem_end$pH[chem_end$treatment==i])}

#CONDUCTIVITY NORMALITY
cond_treatment = c("NS", "SC", "NC", "C")
par(mfrow=c(4, 2))
for(i in cond_treatment) {
  hist(chem_end$Conductivity[chem_end$treatment==i], main=c("Conductivity of ",i))
  b = shapiro.test(chem_end$Conductivity[chem_end$treatment==i])
  legend("topright", legend = b$p.value)
  qqnorm(chem_end$Conductivity[chem_end$treatment==i])
  qqline(chem_end$Conductivity[chem_end$treatment==i])
  print(shapiro.test(chem_end$Conductivity[chem_end$treatment==i]))}

#ALKALINITY NORMALITY
alk_treatment = c("NS", "SC", "NC", "C")
par(mfrow=c(4, 2))
for(i in alk_treatment) {
  hist(chem_end$Alkalinity[chem_end$treatment==i], main=c("Alkalintiy of ",i))
  c = shapiro.test(chem_end$Alkalinity[chem_end$treatment==i])
  legend("topright", legend = c$p.value)
  qqnorm(chem_end$Alkalinity[chem_end$treatment==i])
  qqline(chem_end$Alkalinity[chem_end$treatment==i])
  print(shapiro.test(chem_end$Alkalinity[chem_end$treatment==i]))}

#CALCIUM NORMALITY
ca_treatment = c("NS", "SC", "NC", "C")
par(mfrow=c(4, 2))
for(i in ca_treatment) {
  hist(chem_end$Calcium[chem_end$treatment==i], main=c("Calcium of ",i))
  d = shapiro.test(chem_end$Calcium[chem_end$treatment==i])
  legend("topright", legend = d$p.value)
  qqnorm(chem_end$Calcium[chem_end$treatment==i])
  qqline(chem_end$Calcium[chem_end$treatment==i])
  print(shapiro.test(chem_end$Calcium[chem_end$treatment==i]))}

#----------------SAME CHEM ACROSS TREATMENTS--------------------
num.dates <- as.numeric(chem_end$Date, levels=c("10/12/16", "10/18/16", "10/26/16",
                                                "11/1/16", "11/9/16", "11/15/16",
                                                "11/23/16", "11/29/16"))
chem_end <- data.frame(num.dates, chem_end)
chem_end
str(chem_end)

#PH
```

```
par(mfrow=c(1,1))
par(mar=c(5, 5, 4, 2))
tank<-c("6","9","14","1","5","13","4","10","15","16")
i<-1
plot(x = chem_end$num.dates[chem_end$Tank==tank[i]], y =
chem_end$pH[chem_end$Tank==tank[i]],
    ylim=c(8, 9), xaxt="n", xlab="Date", ylab="pH", pch=i, col=i, cex.lab = 1.5, cex.axis
= 1.5, cex=1.5)
axis(side=1, labels = c(levels(chem_end$Date)), at=c(1:8), cex.axis= 1.5)
for(i in 2:3){
  points(x=chem_end$num.dates[chem_end$Tank==tank[i]],
y=chem_end$pH[chem_end$Tank==tank[i]],
       xaxt="n", xlab="Date", ylab="pH", pch=i, col=1, cex=1.5)
}
for(i in 4:6){
  points(x=chem_end$num.dates[chem_end$Tank==tank[i]],
y=chem_end$pH[chem_end$Tank==tank[i]],
       xaxt="n", xlab="Date", ylab="pH", pch=i, col=2, cex=1.5)
}
for(i in 7:9){
  points(x=chem_end$num.dates[chem_end$Tank==tank[i]],
y=chem_end$pH[chem_end$Tank==tank[i]],
       xaxt="n", xlab="Date", ylab="pH", pch=i, col=3, cex=1.5)
}
for(i in 10){
  points(x=chem_end$num.dates[chem_end$Tank==tank[i]],
y=chem_end$pH[chem_end$Tank==tank[i]],
       xaxt="n", xlab="Date", ylab="pH", pch=i, col=4, cex=1.5)
}
legend(locator(1),tank, pch=1:10, col=c(1, 1, 1, 2, 2, 2, 3, 3, 3, 4), cex = 1.5)    #places
                                                                                    legend where
                                                                                    ever you click on
                                                                                    the plot
legend(locator(1), legend=c("NS", "SC", "NC", "C"), fill=c(1:4), cex= 1.5)

#CONDUCTIVITY
par(mfrow=c(1,1))
tank<-c("6","9","14","1","5","13","4","10","15","16")
i<-1
plot(x = chem_end$num.dates[chem_end$Tank==tank[i]], y =
chem_end$Conductivity[chem_end$Tank==tank[i]],
    ylim=c(4000, 5000), xaxt="n", xlab="Date", ylab="Conductivity", pch=i, col=i, cex=
1.5, cex.lab = 1.5, cex.axis = 1.5)
axis(side=1, labels = c(levels(chem_end$Date)), at=c(1:8), cex.axis=1.5)
for(i in 2:3){
```

```
  points(x=chem_end$num.dates[chem_end$Tank==tank[i]],
y=chem_end$Conductivity[chem_end$Tank==tank[i]],
        xaxt="n", xlab="Date", ylab="Conductivity", pch=i, col=1, cex= 1.5)
}
for(i in 4:6){
  points(x=chem_end$num.dates[chem_end$Tank==tank[i]],
y=chem_end$Conductivity[chem_end$Tank==tank[i]],
        xaxt="n", xlab="Date", ylab="Conductivity", pch=i, col=2, cex= 1.5)
}
for(i in 7:9){
  points(x=chem_end$num.dates[chem_end$Tank==tank[i]],
y=chem_end$Conductivity[chem_end$Tank==tank[i]],
        xaxt="n", xlab="Date", ylab="Conductivity", pch=i, col=3, cex= 1.5)
}
for(i in 10){
  points(x=chem_end$num.dates[chem_end$Tank==tank[i]],
y=chem_end$Conductivity[chem_end$Tank==tank[i]],
        xaxt="n", xlab="Date", ylab="Conductivity", pch=i, col=4, cex= 1.5)
}
legend(locator(1),tank,pch=1:10, col=c(1, 1, 1, 2, 2, 2, 3, 3, 3, 4), cex= 1.5)
legend(locator(1), legend=c("NS", "SC", "NC", "C"), fill=c(1:4), cex= 1.5)

#ALKALINITY
par(mfrow=c(1,1))
tank<-c("6","9","14","1","5","13","4","10","15","16")
i<-1
plot(x = chem_end$num.dates[chem_end$Tank==tank[i]], y =
chem_end$Alkalinity[chem_end$Tank==tank[i]],
    ylim=c(100, 300), xaxt="n", xlab="Date", ylab="Alkalinity", pch=i, col=i, cex= 1.5,
cex.lab = 1.5, cex.axis = 1.5)
axis(side=1, labels = c(levels(chem_end$Date)), at=c(1:8), cex.axis=1.5)
for(i in 2:3){
  points(x=chem_end$num.dates[chem_end$Tank==tank[i]],
y=chem_end$Alkalinity[chem_end$Tank==tank[i]],
        xaxt="n", xlab="Date", ylab="Alkalinity", pch=i, col=1, cex= 1.5)
}
for(i in 4:6){
  points(x=chem_end$num.dates[chem_end$Tank==tank[i]],
y=chem_end$Alkalinity[chem_end$Tank==tank[i]],
        xaxt="n", xlab="Date", ylab="Alkalinity", pch=i, col=2, cex= 1.5)
}
for(i in 7:9){
  points(x=chem_end$num.dates[chem_end$Tank==tank[i]],
y=chem_end$Alkalinity[chem_end$Tank==tank[i]],
        xaxt="n", xlab="Date", ylab="Alkalinity", pch=i, col=3, cex= 1.5)
}
```

```
for(i in 10){
  points(x=chem_end$num.dates[chem_end$Tank==tank[i]],
y=chem_end$Alkalinity[chem_end$Tank==tank[i]],
        xaxt="n", xlab="Date", ylab="Alkalinity", pch=i, col=4, cex= 1.5)
}
legend(locator(1),tank,pch=1:10, col=c(1, 1, 1, 2, 2, 2, 3, 3, 3, 4), cex= 1.5)
legend(locator(1), legend=c("NS", "SC", "NC", "C"), fill=c(1:4), cex= 1.5)

#CALCIUM
par(mfrow=c(1,1))
tank<-c("6","9","14","1","5","13","4","10","15","16")
i<-1
plot(x = chem_end$num.dates[chem_end$Tank==tank[i]], y =
chem_end$Calcium[chem_end$Tank==tank[i]],
    ylim=c(400, 700), xaxt="n", xlab="Date", ylab="Calcium", pch=i, col=i)
axis(side=1, labels = c(levels(chem_end$Date)), at=c(1:8))
for(i in 2:3){
  points(x=chem_end$num.dates[chem_end$Tank==tank[i]],
y=chem_end$Calcium[chem_end$Tank==tank[i]],
        xaxt="n", xlab="Date", ylab="Calcium", pch=i, col=1)
}
for(i in 4:6){
  points(x=chem_end$num.dates[chem_end$Tank==tank[i]],
y=chem_end$Calcium[chem_end$Tank==tank[i]],
        xaxt="n", xlab="Date", ylab="Calcium", pch=i, col=2)
}
for(i in 7:9){
  points(x=chem_end$num.dates[chem_end$Tank==tank[i]],
y=chem_end$Calcium[chem_end$Tank==tank[i]],
        xaxt="n", xlab="Date", ylab="Calcium", pch=i, col=3)
}
for(i in 10){
  points(x=chem_end$num.dates[chem_end$Tank==tank[i]],
y=chem_end$Calcium[chem_end$Tank==tank[i]],
        xaxt="n", xlab="Date", ylab="Calcium", pch=i, col=4)
}
legend(locator(1),tank,pch=1:10, col=c(1, 1, 1, 2, 2, 2, 3, 3, 3, 4))
legend(locator(1), legend=c("NS", "SC", "NC", "C"), fill=c(1:4))

#-----BLOCK EFFECT FOR JUST THE LAST 8 WEEKS AND JUST THE
TREATMENTS USED----------
chem_end
Block <- rep(c("1", "1", "1", "1", "2", "2", "2", "2", "2", "2"),8)     #adding in what block
                                                                        each tank was in and
                                                                        repeating throughout
                                                                        the entire data frame
```

```
Block

chem_end_block <- data.frame(chem_end, Block)
chem_end_block
chem_end_block$treatment <- factor(chem_end_block$treatment, levels = c("SC", "NC",
"NS", "C"))      #ordering the levels of treatments
chem_end_block

#--------------ANOVA, TUKEY TESTS, AND PLOTTING TUKEY TESTS---------------
install.packages("car")
library(car)
install.packages("deSolve")
library(deSolve)

#------pH------
##Type 2 two-way ANOVA's
block_pH <- aov(pH ~ treatment + Block, data=chem_end_block)
Anova(block_pH)
TukeyHSD(block_pH)
#plotting TREATMENT tukey tests
par(mfrow=c(1,2))
par(mar=c(5.1, 5.7, 2, 2))
plot(TukeyHSD(block_pH), cex.axis=2, xlim=c(-0.10, 0.3))

#-------conductivity-------
block_cond <- aov(Conductivity ~ treatment + Block, data=chem_end_block)
Anova(block_cond)
par(mfrow=c(1,2))
par(mar=c(5.1, 5.7, 2, 2))
plot(TukeyHSD(block_cond), cex.axis=2)

#-----alkalinity-----
block_alk <- aov(Alkalinity ~ treatment + Block, data=chem_end_block)
Anova(block_alk)
TukeyHSD(block_alk)
par(mfrow=c(1,1))
par(mar=c(5.1, 5.7, 2, 2))
plot(TukeyHSD(block_alk), cex.axis=2)

#----calcium-----
par(mfrow=c(1,1))
par(mar=c(5.1, 5.7, 2, 2))
block_ca <- aov(Calcium ~ treatment + Block, data=chem_end_block)
Anova(block_ca)
TukeyHSD(block_ca)
```

```
plot(TukeyHSD(block_ca), cex.axis=2)
#-------------boxplots of treatments and blocks-------------
par(mfrow=c(4, 2))
par(mar=c(5, 6, 2, 2) + 0.1)
boxplot(chem_end_block$pH ~ chem_end_block$treatment, cex.axis=1.8,
col=c("gray32", "gray66", "gray91", "white"), ylab="")
mtext(text="pH", line=3.5, cex=1.5, font=2, side=2)
boxplot(chem_end_block$pH ~ chem_end_block$Block, cex.axis=1.8)

par(mar=c(5, 6, 2, 2) + 0.1)
boxplot(chem_end_block$Conductivity ~ chem_end_block$treatment, cex.axis=1.8,
ylab="", font.lab=2, col=c("gray32", "gray66", "gray91", "white"))
mtext(text=expression(bold(paste("Conductivity (", mu, "S/cm")))), line=3.5, cex=1.5,
font=2, side=2)
boxplot(chem_end_block$Conductivity ~ chem_end_block$Block, cex.axis=1.8)

par(mar=c(5, 6, 2, 2) + 0.1)
boxplot(chem_end_block$Alkalinity ~ chem_end_block$treatment, cex.axis=1.8,
ylab="", font.lab=2, col=c("gray32", "gray66", "gray91", "white"))
mtext(text=expression(bold("Alkalinity "("ppm CaCO"^"3"))), line=3.5, cex=1.5, font=2,
side=2)
boxplot(chem_end_block$Alkalinity ~ chem_end_block$Block, cex.axis=1.8)

par(mar=c(5.3, 6, 2, 2) + 0.1)
boxplot(chem_end_block$Calcium ~ chem_end_block$treatment, cex.axis=1.8,
cex.lab=2.3, xlab="", font.lab=2, ylab="", col=c("gray32", "gray66", "gray91", "white"))
mtext(text="Calcium (ppm)", line=3.5, cex=1.5, font=2, side=2)
mtext(text="Treatment", line= 3.4, cex=1.5, font=2, side=1)
boxplot(chem_end_block$Calcium ~ chem_end_block$Block, cex.axis=1.8, xlab="",
font.lab=2, cex.lab=2.3)
mtext(text="Block", line= 3.4, cex=1.5, font=2, side=1)

#------------additional analyses of means and normalities------------
mean(chem_end_block$pH[chem_end_block$treatment=="SC"])
mean(chem_end_block$pH[chem_end_block$treatment=="NC"])
mean(chem_end_block$pH[chem_end_block$treatment=="NS"])
mean(chem_end_block$pH[chem_end_block$treatment=="C"])

mean(chem_end_block$Alkalinity[chem_end_block$treatment=="SC"])
mean(chem_end_block$Alkalinity[chem_end_block$treatment=="NC"])
mean(chem_end_block$Alkalinity[chem_end_block$treatment=="NS"])
mean(chem_end_block$Alkalinity[chem_end_block$treatment=="C"])

shapiro.test(chem_end_block$Conductivity[chem_end_block$treatment=="SC"])
shapiro.test(chem_end_block$Conductivity[chem_end_block$treatment=="NC"])
shapiro.test(chem_end_block$Conductivity[chem_end_block$treatment=="NS"])
```

```
summary(chem_end_block$Conductivity[chem_end_block$treatment=="SC"])
summary(chem_end_block$Conductivity[chem_end_block$treatment=="NC"])
summary(chem_end_block$Conductivity[chem_end_block$treatment=="NS"])
mean(chem_end_block$Conductivity[chem_end_block$treatment=="SC"])
mean(chem_end_block$Conductivity[chem_end_block$treatment=="NC"])
mean(chem_end_block$Conductivity[chem_end_block$treatment=="NS"])

sort(chem_end_block$pH)
sort(chem_end_block$Conductivity)

mean(chem_date$Temp)
mean(chem_date$Salinity, na.rm=TRUE)
```

Algal Growth

```
#----------------------AVG GROWTH PER STRAIN PER TREATMENT----------------------
#import average growth per strain
file.choose(new=TRUE)
avg_grow = read.csv([filename])     #import csv file with the average 480 nm wavelength
fluorescence data for each tube
avg_grow
avg_grow$Date = factor(avg_grow$Date, levels=c("2/22/17", "2/24/17",
                                               "2/26/17", "2/28/17",
                                               "3/2/17", "3/4/17",
                                               "3/6/17", "3/8/17",
                                               "3/10/17"))      ##ordering the dates
str(avg_grow)

num.dates <- as.numeric(avg_grow$Date, levels=c("2/22/17", "2/24/17",
                                               "2/26/17", "2/28/17",
                                               "3/2/17", "3/4/17",
                                               "3/6/17", "3/8/17",
                                               "3/10/17"))    #number the dates of
                                          data collection for graphing purposes
avg_grow <- data.frame(num.dates, avg_grow)

#--------------plot avg growth per strain per treatment---------------
#PRE STRAINS
Pre <- c("Pre 1984", "Pre 3430", "Pre 1015", "Pre 374", "Pre Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_grow$num.dates[avg_grow$Tube==Pre[i]],
y=avg_grow$Avg.F.480[avg_grow$Tube==Pre[i]], main=Pre[i],
       ylim=c(-10, 400), xaxt="n", xlab="Date", ylab="F", lty=1)
  axis(side=1, labels = c(levels(avg_grow$Date)), at=c(1:9))}
```

```
#CONTROL 2 STRAINS
Control_2 <- c("Control 2-1984", "Control 2-3430", "Control 2-1015", "Control 2-374",
"Control 2-Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_grow$num.dates[avg_grow$Tube==Control_2[i]],
y=avg_grow$Avg.F.480[avg_grow$Tube==Control_2[i]], main=Control_2[i], ylim=c(-
10, 300), xaxt="n", xlab="Date", ylab="F")
  axis(side=1, labels = c(levels(avg_grow$Date)), at=c(1:9))}

#CONTROL 3 STRAINS
Control_3 <- c("Control 3-1984", "Control 3-3430", "Control 3-1015", "Control 3-374",
"Control 3-Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_grow$num.dates[avg_grow$Tube==Control_3[i]],
y=avg_grow$Avg.F.480[avg_grow$Tube==Control_3[i]], main=Control_3[i], ylim=c(-
10, 300), xaxt="n", xlab="Date", ylab="F")
  axis(side=1, labels = c(levels(avg_grow$Date)), at=c(1:9))}

#NC 2 STRAINS
NC_2 <- c("NC 2-1984", "NC 2-3430", "NC 2-1015", "NC 2-374", "NC 2-Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_grow$num.dates[avg_grow$Tube==NC_2[i]],
y=avg_grow$Avg.F.480[avg_grow$Tube==NC_2[i]], main=NC_2[i], ylim=c(-10, 350),
xaxt="n", xlab="Date", ylab="F")
  axis(side=1, labels = c(levels(avg_grow$Date)), at=c(1:9))}

#NC 3 STRAINS
NC_3 <- c("NC 3-1984", "NC 3-3430", "NC 3-1015", "NC 3-374", "NC 3-Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_grow$num.dates[avg_grow$Tube==NC_3[i]],
y=avg_grow$Avg.F.480[avg_grow$Tube==NC_3[i]], main=NC_3[i], ylim=c(-10, 300),
xaxt="n", xlab="Date", ylab="F")
  axis(side=1, labels = c(levels(avg_grow$Date)), at=c(1:9))}

#NS 2 STRAINS
NS_2 <- c("NS 2-1984", "NS 2-3430", "NS 2-1015", "NS 2-374", "NS 2-Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_grow$num.dates[avg_grow$Tube==NS_2[i]],
y=avg_grow$Avg.F.480[avg_grow$Tube==NS_2[i]], main=NS_2[i], ylim=c(-10, 300),
xaxt="n", xlab="Date", ylab="F")
  axis(side=1, labels = c(levels(avg_grow$Date)), at=c(1:9))}
```

```
#NS 3 STRAINS
NS_3 <- c("NS 3-1984", "NS 3-3430", "NS 3-1015", "NS 3-374", "NS 3-Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_grow$num.dates[avg_grow$Tube==NS_3[i]],
y=avg_grow$Avg.F.480[avg_grow$Tube==NS_3[i]], main=NS_3[i], ylim=c(-10, 300),
xaxt="n", xlab="Date", ylab="F")
  axis(side=1, labels = c(levels(avg_grow$Date)), at=c(1:9))}

#SC 2 STRAINS
SC_2 <- c("SC 2-1984", "SC 2-3430", "SC 2-1015", "SC 2-374", "SC 2-Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_grow$num.dates[avg_grow$Tube==SC_2[i]],
y=avg_grow$Avg.F.480[avg_grow$Tube==SC_2[i]], main=SC_2[i], ylim=c(-10, 300),
xaxt="n", xlab="Date", ylab="F")
  axis(side=1, labels = c(levels(avg_grow$Date)), at=c(1:9))}

#SC 3 STRAINS
SC_3 <- c("SC 3-1984", "SC 3-3430", "SC 3-1015", "SC 3-374", "SC 3-Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_grow$num.dates[avg_grow$Tube==SC_3[i]],
y=avg_grow$Avg.F.480[avg_grow$Tube==SC_3[i]], main=SC_3[i], ylim=c(-10, 300),
xaxt="n", xlab="Date", ylab="F")
  axis(side=1, labels = c(levels(avg_grow$Date)), at=c(1:9))}

#--------------------FV/FM OVER TIME OF EACH STRAIN-------------------------------------
file.choose(new=TRUE)
avg_fvfm = read.csv([filename])      #import csv file for average Fv/Fm for 480 nm
wavelength for each tube
avg_fvfm
avg_fvfm$Date = factor(avg_fvfm$Date, levels=c("2/22/17", "2/24/17",
                                    "2/26/17", "2/28/17",
                                    "3/2/17", "3/4/17",
                                    "3/6/17", "3/8/17",
                                    "3/10/17"))      ###ordering the dates
str(avg_fvfm)

num.dates.fvfm <- as.numeric(avg_fvfm$Date, levels=c("2/22/17", "2/24/17",
                                      "2/26/17", "2/28/17",
                                      "3/2/17", "3/4/17",
                                      "3/6/17", "3/8/17",
                                      "3/10/17"))    ###numbering the dates for graphing
purposes
avg_fvfm <- data.frame(num.dates.fvfm, avg_fvfm)
```

```
#plot avg growth per strain per treatment
#PRE STRAINS
Pre.fvfm <- c("Pre 1984", "Pre 3430", "Pre 1015", "Pre 374", "Pre Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==Pre.fvfm[i]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==Pre.fvfm[i]], main=Pre.fvfm[i],
ylim=c(0, 1), xaxt="n", xlab="Date", ylab="Fv/Fm", lty=1)
  axis(side=1, labels = c(levels(avg_fvfm$Date)), at=c(1:9))}

#CONTROL 2 STRAINS
Control_2.fvfm <- c("Control 2-1984", "Control 2-3430", "Control 2-1015", "Control 2-
374", "Control 2-Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==Control_2.fvfm[i]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==Control_2.fvfm[i]],
main=Control_2.fvfm[i], ylim=c(0, 1), xaxt="n", xlab="Date", ylab="Fv/Fm")
  axis(side=1, labels = c(levels(avg_fvfm$Date)), at=c(1:9))}

#CONTROL 3 STRAINS
Control_3.fvfm <- c("Control 3-1984", "Control 3-3430", "Control 3-1015", "Control 3-
374", "Control 3-Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==Control_3.fvfm[i]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==Control_3.fvfm[i]],
main=Control_3.fvfm[i], ylim=c(0, 1), xaxt="n", xlab="Date", ylab="Fv/Fm")
  axis(side=1, labels = c(levels(avg_fvfm$Date)), at=c(1:9))}

#NC 2 STRAINS
NC_2.fvfm <- c("NC 2-1984", "NC 2-3430", "NC 2-1015", "NC 2-374", "NC 2-Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==NC_2.fvfm[i]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==NC_2.fvfm[i]], main=NC_2.fvfm[i],
ylim=c(0, 1), xaxt="n", xlab="Date", ylab="Fv/Fm")
  axis(side=1, labels = c(levels(avg_fvfm$Date)), at=c(1:9))}

#NC 3 STRAINS
NC_3.fvfm <- c("NC 3-1984", "NC 3-3430", "NC 3-1015", "NC 3-374", "NC 3-Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==NC_3.fvfm[i]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==NC_3.fvfm[i]], main=NC_3.fvfm[i],
ylim=c(0, 1), xaxt="n", xlab="Date", ylab="Fv/Fm")
```

```
  axis(side=1, labels = c(levels(avg_fvfm$Date)), at=c(1:9))}

#NS 2 STRAINS
NS_2.fvfm <- c("NS 2-1984", "NS 2-3430", "NS 2-1015", "NS 2-374", "NS 2-Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==NS_2.fvfm[i]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==NS_2.fvfm[i]], main=NS_2.fvfm[i],
ylim=c(0, 1), xaxt="n", xlab="Date", ylab="Fv/Fm")
  axis(side=1, labels = c(levels(avg_fvfm$Date)), at=c(1:9))}

#NS 3 STRAINS
NS_3.fvfm <- c("NS 3-1984", "NS 3-3430", "NS 3-1015", "NS 3-374", "NS 3-Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==NS_3.fvfm[i]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==NS_3.fvfm[i]], main=NS_3.fvfm[i],
ylim=c(0, 1), xaxt="n", xlab="Date", ylab="Fv/Fm")
  axis(side=1, labels = c(levels(avg_fvfm$Date)), at=c(1:9))}

#SC 2 STRAINS
SC_2.fvfm <- c("SC 2-1984", "SC 2-3430", "SC 2-1015", "SC 2-374", "SC 2-Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==SC_2.fvfm[i]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==SC_2.fvfm[i]], main=SC_2.fvfm[i],
ylim=c(0, 1), xaxt="n", xlab="Date", ylab="Fv/Fm")
  axis(side=1, labels = c(levels(avg_fvfm$Date)), at=c(1:9))}

#SC 3 STRAINS
SC_3.fvfm <- c("SC 3-1984", "SC 3-3430", "SC 3-1015", "SC 3-374", "SC 3-Nano")
par(mfrow=c(3, 2))
for(i in 1:5) {
  plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==SC_3.fvfm[i]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==SC_3.fvfm[i]], main=SC_3.fvfm[i],
ylim=c(0, 1), xaxt="n", xlab="Date", ylab="Fv/Fm")
  axis(side=1, labels = c(levels(avg_fvfm$Date)), at=c(1:9))}

###----------------------COMPARING F and Fv/Fm FOR ALL STRAINS----------------------
##--------------------1984------------
combo_1984 <- c("Pre 1984", "Control 2-1984", "Control 3-1984", "SC 2-1984", "SC 3-
1984", "NC 2-1984", "NC 3-1984", "NS 2-1984", "NS 3-1984")
leg_1984 <- c("Pre", "Mid Control", "End Control", "Mid SC", "End SC", "Mid NC",
"End NC", "Mid NS", "End NS")
par(mfrow=c(3, 3))
par(mar = c(1.5, 5.5, 5, 3))
```

```
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1984[1]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1984[1]],
    ylim=c(-10, 100), xaxt="n", xlab="", ylab="F", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1984[1]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1984[1]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_1984[1], bty="n", cex=2)
legend(locator(1), pch=c(16, NA), lty=c(1, 2), col=c("black", "red"), legend = c("F",
"Fv/Fm"), cex=2.2, bty="n", lwd=2)
#-------------
par(mar = c(1.5, 3.3, 5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1984[2]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1984[2]],
    ylim=c(-10, 100), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1984[2]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1984[2]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_1984[2], bty="n", cex=2)
#-------------
par(mar = c(1.5, 3, 5, 5.3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1984[3]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1984[3]],
    ylim=c(-10, 100), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1984[3]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1984[3]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
mtext("Fv/Fm", side=4, line=3.5, cex=1.5, font=2, col=2)
legend(locator(1), legend = leg_1984[3], bty="n", cex=2)

#---------------------------------
par(mar = c(2.5, 5.5, 2.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1984[4]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1984[4]],
```

```
    ylim=c(-10, 100), xaxt="n", xlab="", ylab="F", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1984[4]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1984[4]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_1984[4], bty="n", cex=2)
#-------------
par(mar = c(2.5, 3.3, 2.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1984[5]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1984[5]],
    ylim=c(-10, 100), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1984[5]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1984[5]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_1984[5], bty="n", cex=2)
#-------------
par(mar = c(2.5, 3, 2.5, 5.3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1984[6]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1984[6]],
    ylim=c(-10, 100), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1984[6]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1984[6]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
mtext("Fv/Fm", side=4, line=3.5, cex=1.5, font=2, col=2)
legend(locator(1), legend = leg_1984[6], bty="n", cex=2)

#-------------
par(mar = c(5, 5.5, 1.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1984[7]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1984[7]],
    ylim=c(-10, 100), xaxt="n", xlab="", ylab="F", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
mtext("Date", side=1, line=4, cex=1.5, font=2)
axis(side=1, labels = c("2/22", "2/26", "3/2","3/6","3/10"), at=c(1, 3, 5, 7, 9),
cex.axis=2.2)
```

```
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1984[7]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1984[7]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_1984[7], bty="n", cex=2)
#-------------
par(mar = c(5, 3.3, 1.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1984[8]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1984[8]],
    ylim=c(-10, 100), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
mtext("Date", side=1, line=4, cex=1.5, font=2)
axis(side=1, labels = c("2/22", "2/26", "3/2","3/6","3/10"), at=c(1, 3, 5, 7, 9),
cex.axis=2.2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1984[8]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1984[8]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_1984[8], bty="n", cex=2)
#-------------
par(mar = c(5, 3, 1.5, 5.3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1984[9]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1984[9]],
    ylim=c(-10, 100), xaxt="n", xlab="", ylab="", lty=1, pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
mtext("Date", side=1, line=4, cex=1.5, font=2)
axis(side=1, labels = c("2/22", "2/26", "3/2","3/6","3/10"), at=c(1, 3, 5, 7, 9),
cex.axis=2.2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1984[9]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1984[9]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
mtext("Fv/Fm", side=4, line=3.5, cex=1.5, font=2, col=2)
legend(locator(1), legend = leg_1984[9], bty="n", cex=2)

##-------------3430---------
combo_3430 <- c("Pre 3430", "Control 2-3430", "Control 3-3430", "SC 2-3430", "SC 3-
3430", "NC 2-3430", "NC 3-3430", "NS 2-3430", "NS 3-3430")
leg_3430 <- c("Pre", "Mid Control", "End Control", "Mid SC", "End SC", "Mid NC",
"End NC", "Mid NS", "End NS")
```

```
par(mfrow=c(3, 3))
par(mar = c(1.5, 5.5, 5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_3430[1]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_3430[1]],
    ylim=c(-10, 80), xaxt="n", xlab="", ylab="F", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_3430[1]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_3430[1]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_3430[1], bty="n", cex=2)
legend(locator(1), pch=c(16, NA), lty=c(1, 2), col=c("black", "red"), legend = c("F",
"Fv/Fm"), cex=2.2, bty="n", lwd=2)
#-------------
par(mar = c(1.5, 3.3, 5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_3430[2]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_3430[2]],
    ylim=c(-10, 80), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_3430[2]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_3430[2]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_3430[2], bty="n", cex=2)
#-------------
par(mar = c(1.5, 3, 5, 5.3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_3430[3]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_3430[3]],
    ylim=c(-10, 80), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_3430[3]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_3430[3]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
mtext("Fv/Fm", side=4, line=3.5, cex=1.5, font=2, col=2)
legend(locator(1), legend = leg_3430[3], bty="n", cex=2)
```

```
#---------------------------------
par(mar = c(2.5, 5.5, 2.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_3430[4]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_3430[4]],
    ylim=c(-10, 80), xaxt="n", xlab="", ylab="F", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_3430[4]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_3430[4]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_3430[4], bty="n", cex=2)
#-------------
par(mar = c(2.5, 3.3, 2.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_3430[5]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_3430[5]],
    ylim=c(-10, 80), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_3430[5]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_3430[5]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_3430[5], bty="n", cex=2)
#-------------
par(mar = c(2.5, 3, 2.5, 5.3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_3430[6]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_3430[6]],
    ylim=c(-10, 80), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_3430[6]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_3430[6]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
mtext("Fv/Fm", side=4, line=3.5, cex=1.5, font=2, col=2)
legend(locator(1), legend = leg_3430[6], bty="n", cex=2)

#-------------
par(mar = c(5, 5.5, 1.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_3430[7]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_3430[7]],
```

```
    ylim=c(-10, 80), xaxt="n", xlab="", ylab="F", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
mtext("Date", side=1, line=4, cex=1.5, font=2)
axis(side=1, labels = c("2/22", "2/26", "3/2","3/6","3/10"), at=c(1, 3, 5, 7, 9),
cex.axis=2.2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_3430[7]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_3430[7]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_3430[7], bty="n", cex=2)
#-------------
par(mar = c(5, 3.3, 1.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_3430[8]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_3430[8]],
    ylim=c(-10, 80), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
mtext("Date", side=1, line=4, cex=1.5, font=2)
axis(side=1, labels = c("2/22", "2/26", "3/2","3/6","3/10"), at=c(1, 3, 5, 7, 9),
cex.axis=2.2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_3430[8]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_3430[8]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_3430[8], bty="n", cex=2)
#-------------
par(mar = c(5, 3, 1.5, 5.3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_3430[9]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_3430[9]],
    ylim=c(-10, 80), xaxt="n", xlab="", ylab="", lty=1, pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
mtext("Date", side=1, line=4, cex=1.5, font=2)
axis(side=1, labels = c("2/22", "2/26", "3/2","3/6","3/10"), at=c(1, 3, 5, 7, 9),
cex.axis=2.2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_3430[9]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_3430[9]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
mtext("Fv/Fm", side=4, line=3.5, cex=1.5, font=2, col=2)
legend(locator(1), legend = leg_3430[9], bty="n", cex=2)
```

```
##-----------------1015-------------------
combo_1015 <- c("Pre 1015", "Control 2-1015", "Control 3-1015", "SC 2-1015", "SC 3-
1015", "NC 2-1015", "NC 3-1015", "NS 2-1015", "NS 3-1015")
leg_1015 <- c("Pre", "Mid Control", "End Control", "Mid SC", "End SC", "Mid NC",
"End NC", "Mid NS", "End NS")
par(mfrow=c(3, 3))
par(mar = c(1.5, 5.5, 5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1015[1]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1015[1]],
    ylim=c(-10, 300), xaxt="n", xlab="", ylab="F", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1015[1]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1015[1]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_1015[1], bty="n", cex=2)
legend(locator(1), pch=c(16, NA), lty=c(1, 2), col=c("black", "red"), legend = c("F",
"Fv/Fm"), cex=2.2, bty="n", lwd=2)
#-------------
par(mar = c(1.5, 3.3, 5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1015[2]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1015[2]],
    ylim=c(-10, 300), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1015[2]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1015[2]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_1015[2], bty="n", cex=2)
#-------------
par(mar = c(1.5, 3, 5, 5.3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1015[3]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1015[3]],
    ylim=c(-10, 300), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1015[3]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1015[3]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
mtext("Fv/Fm", side=4, line=3.5, cex=1.5, font=2, col=2)
```

```
legend(locator(1), legend = leg_1015[3], bty="n", cex=2)

#---------------------------------
par(mar = c(2.5, 5.5, 2.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1015[4]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1015[4]],
    ylim=c(-10, 300), xaxt="n", xlab="", ylab="F", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1015[4]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1015[4]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_1015[4], bty="n", cex=2)
#-------------
par(mar = c(2.5, 3.3, 2.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1015[5]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1015[5]],
    ylim=c(-10, 300), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1015[5]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1015[5]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_1015[5], bty="n", cex=2)
#-------------
par(mar = c(2.5, 3, 2.5, 5.3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1015[6]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1015[6]],
    ylim=c(-10, 300), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1015[6]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1015[6]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
mtext("Fv/Fm", side=4, line=3.5, cex=1.5, font=2, col=2)
legend(locator(1), legend = leg_1015[6], bty="n", cex=2)
```

```
#-------------
par(mar = c(5, 5.5, 1.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1015[7]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1015[7]],
    ylim=c(-10, 300), xaxt="n", xlab="", ylab="F", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
mtext("Date", side=1, line=4, cex=1.5, font=2)
axis(side=1, labels = c("2/22", "2/26", "3/2","3/6","3/10"), at=c(1, 3, 5, 7, 9),
cex.axis=2.2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1015[7]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1015[7]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_1015[7], bty="n", cex=2)
#-------------
par(mar = c(5, 3.3, 1.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1015[8]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1015[8]],
    ylim=c(-10, 300), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
mtext("Date", side=1, line=4, cex=1.5, font=2)
axis(side=1, labels = c("2/22", "2/26", "3/2","3/6","3/10"), at=c(1, 3, 5, 7, 9),
cex.axis=2.2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1015[8]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1015[8]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_1015[8], bty="n", cex=2)
#-------------
par(mar = c(5, 3, 1.5, 5.3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_1015[9]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_1015[9]],
    ylim=c(-10, 300), xaxt="n", xlab="", ylab="", lty=1, pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
mtext("Date", side=1, line=4, cex=1.5, font=2)
axis(side=1, labels = c("2/22", "2/26", "3/2","3/6","3/10"), at=c(1, 3, 5, 7, 9),
cex.axis=2.2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_1015[9]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_1015[9]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
```

```
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
mtext("Fv/Fm", side=4, line=3.5, cex=1.5, font=2, col=2)
legend(locator(1), legend = leg_1015[9], bty="n", cex=2)

##---------------------374-----------
combo_374 <- c("Pre 374", "Control 2-374", "Control 3-374", "SC 2-374", "SC 3-374",
"NC 2-374", "NC 3-374", "NS 2-374", "NS 3-374")
leg_374 <- c("Pre", "Mid Control", "End Control", "Mid SC", "End SC", "Mid NC",
"End NC", "Mid NS", "End NS")
par(mfrow=c(3, 3))
par(mar = c(1.5, 5.5, 5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_374[1]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_374[1]],
    ylim=c(-10, 200), xaxt="n", xlab="", ylab="F", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_374[1]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_374[1]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_374[1], bty="n", cex=2)
legend(locator(1), pch=c(16, NA), lty=c(1, 2), col=c("black", "red"), legend = c("F",
"Fv/Fm"), cex=2.2, bty="n", lwd=2)
#-------------
par(mar = c(1.5, 3.3, 5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_374[2]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_374[2]],
    ylim=c(-10, 200), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_374[2]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_374[2]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_374[2], bty="n", cex=2)
#-------------
par(mar = c(1.5, 3, 5, 5.3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_374[3]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_374[3]],
    ylim=c(-10, 200), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_374[3]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_374[3]],
```

```
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
mtext("Fv/Fm", side=4, line=3.5, cex=1.5, font=2, col=2)
legend(locator(1), legend = leg_374[3], bty="n", cex=2)

#---------------------------------
par(mar = c(2.5, 5.5, 2.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_374[4]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_374[4]],
    ylim=c(-10, 200), xaxt="n", xlab="", ylab="F", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_374[4]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_374[4]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_374[4], bty="n", cex=2)
#-------------
par(mar = c(2.5, 3.3, 2.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_374[5]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_374[5]],
    ylim=c(-10, 200), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_374[5]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_374[5]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_374[5], bty="n", cex=2)
#-------------
par(mar = c(2.5, 3, 2.5, 5.3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_374[6]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_374[6]],
    ylim=c(-10, 200), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_374[6]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_374[6]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
mtext("Fv/Fm", side=4, line=3.5, cex=1.5, font=2, col=2)
legend(locator(1), legend = leg_374[6], bty="n", cex=2)
```

```
#-------------
par(mar = c(5, 5.5, 1.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_374[7]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_374[7]],
    ylim=c(-10, 200), xaxt="n", xlab="", ylab="F", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
mtext("Date", side=1, line=4, cex=1.5, font=2)
axis(side=1, labels = c("2/22", "2/26", "3/2","3/6","3/10"), at=c(1, 3, 5, 7, 9),
cex.axis=2.2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_374[7]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_374[7]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_374[7], bty="n", cex=2)
#-------------
par(mar = c(5, 3.3, 1.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_374[8]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_374[8]],
    ylim=c(-10, 200), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
mtext("Date", side=1, line=4, cex=1.5, font=2)
axis(side=1, labels = c("2/22", "2/26", "3/2","3/6","3/10"), at=c(1, 3, 5, 7, 9),
cex.axis=2.2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_374[8]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_374[8]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_374[8], bty="n", cex=2)
#-------------
par(mar = c(5, 3, 1.5, 5.3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_374[9]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_374[9]],
    ylim=c(-10, 200), xaxt="n", xlab="", ylab="", lty=1, pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
mtext("Date", side=1, line=4, cex=1.5, font=2)
axis(side=1, labels = c("2/22", "2/26", "3/2","3/6","3/10"), at=c(1, 3, 5, 7, 9),
cex.axis=2.2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_374[9]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_374[9]],
```

```
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
mtext("Fv/Fm", side=4, line=3.5, cex=1.5, font=2, col=2)
legend(locator(1), legend = leg_374[9], bty="n", cex=2)

##--------------------Nano-----------
combo_Nano <- c("Pre Nano", "Control 2-Nano", "Control 3-Nano", "SC 2-Nano", "SC
3-Nano", "NC 2-Nano", "NC 3-Nano", "NS 2-Nano", "NS 3-Nano")
leg_Nano <- c("Pre", "Mid Control", "End Control", "Mid SC", "End SC", "Mid NC",
"End NC", "Mid NS", "End NS")
par(mfrow=c(3, 3))
par(mar = c(1.5, 5.5, 5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_Nano[1]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_Nano[1]],
    ylim=c(-10, 400), xaxt="n", xlab="", ylab="F", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_Nano[1]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_Nano[1]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_Nano[1], bty="n", cex=2)
legend(locator(1), pch=c(16, NA), lty=c(1, 2), col=c("black", "red"), legend = c("F",
"Fv/Fm"), cex=2.2, bty="n", lwd=2)
#-------------
par(mar = c(1.5, 3.3, 5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_Nano[2]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_Nano[2]],
    ylim=c(-10, 400), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_Nano[2]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_Nano[2]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_Nano[2], bty="n", cex=2)
#-------------
par(mar = c(1.5, 3, 5, 5.3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_Nano[3]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_Nano[3]],
    ylim=c(-10, 400), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
```

```
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_Nano[3]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_Nano[3]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
mtext("Fv/Fm", side=4, line=3.5, cex=1.5, font=2, col=2)
legend(locator(1), legend = leg_Nano[3], bty="n", cex=2)

#---------------------------------
par(mar = c(2.5, 5.5, 2.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_Nano[4]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_Nano[4]],
    ylim=c(-10, 400), xaxt="n", xlab="", ylab="F", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_Nano[4]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_Nano[4]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_Nano[4], bty="n", cex=2)
#-------------
par(mar = c(2.5, 3.3, 2.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_Nano[5]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_Nano[5]],
    ylim=c(-10, 400), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_Nano[5]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_Nano[5]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_Nano[5], bty="n", cex=2)
#-------------
par(mar = c(2.5, 3, 2.5, 5.3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_Nano[6]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_Nano[6]],
    ylim=c(-10, 400), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_Nano[6]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_Nano[6]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
```

```
mtext("Fv/Fm", side=4, line=3.5, cex=1.5, font=2, col=2)
legend(locator(1), legend = leg_Nano[6], bty="n", cex=2)

#-------------
par(mar = c(5, 5.5, 1.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_Nano[7]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_Nano[7]],
    ylim=c(-10, 400), xaxt="n", xlab="", ylab="F", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
mtext("Date", side=1, line=4, cex=1.5, font=2)
axis(side=1, labels = c("2/22", "2/26", "3/2","3/6","3/10"), at=c(1, 3, 5, 7, 9),
cex.axis=2.2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_Nano[7]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_Nano[7]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_Nano[7], bty="n", cex=2)
#-------------
par(mar = c(5, 3.3, 1.5, 3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_Nano[8]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_Nano[8]],
    ylim=c(-10, 400), xaxt="n", xlab="", ylab="", pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
mtext("Date", side=1, line=4, cex=1.5, font=2)
axis(side=1, labels = c("2/22", "2/26", "3/2","3/6","3/10"), at=c(1, 3, 5, 7, 9),
cex.axis=2.2)
par(new=TRUE)
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_Nano[8]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_Nano[8]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
legend(locator(1), legend = leg_Nano[8], bty="n", cex=2)
#-------------
par(mar = c(5, 3, 1.5, 5.3))
plot(x=avg_grow$num.dates[avg_grow$Tube==combo_Nano[9]],
y=avg_grow$Avg.F.480[avg_grow$Tube==combo_Nano[9]],
    ylim=c(-10, 400), xaxt="n", xlab="", ylab="", lty=1, pch=16, col=1, cex.lab=2.5,
cex.axis=2.2, cex=2, font.lab=2, type="b", lwd=2)
mtext("Date", side=1, line=4, cex=1.5, font=2)
axis(side=1, labels = c("2/22", "2/26", "3/2","3/6","3/10"), at=c(1, 3, 5, 7, 9),
cex.axis=2.2)
par(new=TRUE)
```

```
plot(x=avg_fvfm$num.dates.fvfm[avg_fvfm$Tube==combo_Nano[9]],
y=avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Tube==combo_Nano[9]],
    ylim=c(0, 1), xaxt="n", type="b", axes = FALSE, bty = "n", xlab = "", ylab = "",
col=c("red"), lty=2, lwd=2)
axis(side=4, at = c(0, 0.2, 0.4, 0.6, 0.8, 1), cex.axis=2.2)
mtext("Fv/Fm", side=4, line=3.5, cex=1.5, font=2, col=2)
legend(locator(1), legend = leg_Nano[9], bty="n", cex=2)


#-------------------ranges of fluorescence and Fv/Fm values----------------------
avg_grow
Strain = rep(c("1984", "3430", "1015", "374", "Nano"), 99)       #adding a column with
what each tube contained
Strain
avg_grow <- data.frame(avg_grow, Strain)
avg_grow
sort(avg_grow$Avg.F.480[avg_grow$Strain=="1984"])             #getting a range of
values for each strain
sort(avg_grow$Avg.F.480[avg_grow$Strain=="3430"])
sort(avg_grow$Avg.F.480[avg_grow$Strain=="1015"])
sort(avg_grow$Avg.F.480[avg_grow$Strain=="374"])
sort(avg_grow$Avg.F.480[avg_grow$Strain=="Nano"])


avg_fvfm
Strain = rep(c("1984", "3430", "1015", "374", "Nano"), 99)       #adding a column with
what each tube contained
Strain
avg_fvfm <- data.frame(avg_fvfm, Strain)
avg_fvfm
sort(avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Strain=="1984"])         #getting a range of
values for each strain
sort(avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Strain=="3430"])
sort(avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Strain=="1015"])
sort(avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Strain=="374"])
sort(avg_fvfm$Avg..Fv.Fm.480[avg_fvfm$Strain=="Nano"])
```

References

Armbrust, E.V., J.A. Berges, C. Bowler, B.R. Green, D. Martinez, N.H. Putnam, S. Zhou, A.E. Allen, K.E. Apt, M. Bechner, M.A. Brzezinski, B.K. Chaal, A. Chiovitti, A.K. Davis, M.S. Demarest, J.C. Detter, T. Glavina, D. Goodstein, M.Z. Hadi, U. Hellsten, M. Hildebrand, B.D. Jenkins, J. Jurka, V.V. Kapitonov, N. Kroger, W.W.Y. Lau, T.W. Lane, F.W. Larimer, J.C. Lippmeier, S. Lucas, M. Medina, A. Montsant, M. Obornik, M.S. Parker, B. Palenik, G.J. Pazour, P.M. Richardson, T.A. Rynearson, M.A. Saito, D.C. Schwartz, K. Thamatrakoln, K. Valentin, A. Vardi, F.P. Wilkerson, and D.S. Rokhsar. 2004. The Genome of the Diatom *Thalassiosira pseudonana*: Ecology, Evolution, and Metabolism. Science 306:79-86.

Arnold, S.J., M.J. Sellars, P.J. Crocos, and G.J. Coman. 2006. Intensive production of juvenile tiger shrimp *Penaeus monodon:* An evaluation of stocking density and artificial substrates. Aquaculture 261: 890-896.

Azim, M.E., A. Milstein, M.A. Wahab, M.C.J. Verdegam. 2003. Periphyotn-water quality relationships in fertilized fishponds with artificial substrates. Aquaculture 228: 169-187.

Azim, M.E., M.A. Wahab, M.C.J. Verdegem, A.A. van Dam, J.M. van Rooij, and M.C.M. Beveridge. 2002. The effects of artificial substrates on freshwater pond productivity and water quality and the implications for periphyton-based aquaculture. Aquatic Living Resources 15: 231-241.

Bahr, L.M. and W.P. Lanier. 1981. The Ecology of Intertidal Oyster Reefs of the South Atlantic Coast: a Community Profile. U.S. Fish and Wildlife Service, Blot. Program, Washington D.C. FWS/OBS 81/15. 105 pp.

Bartol, I.K., R. Mann, and M. Luckenbach. 1999. Growth and mortality of oysters (*Crassostrea virginica*) on constructed intertidal reefs: effects of tidal height and substrate level. Journal of Experimental Marine Biology and Ecology. 237: 157-184.

Beaufort L., I. Probert , T. de Garidel-Thoron1 , E. M. Bendif , D. Ruiz-Pino , N. Metzl , C. Goye4 , N. Buchet , P. Coupel , M. Grelaud, B. Rost , R. E. M. Rickaby, and C. de Vargas. 2011. Sensitivity of coccolithophores to carbonate chemistry and ocean acidification. Nature 476: 80-83.

Beck, M.W., R. D. Brumbaugh, L. Airoldi, A. Carranza, L. D. Coen, C. Crawford, O. Defeo, G. J. Edgar, B. Hancock, M. C. Kay, H. S. Lenihan, M. W. Luckenbach, C. L. Toropova, G. Zhang, and X. Guo. 2011. Oyster Reefs at Risk and Recommendations for Conservation, Restoration, and Management. BioScience 61:107-116.

Beebee, T.J.C. 1983. Habitat Selection by amphibians across an agricultural land-heathland transect in Britain. Biological Conservation 27(2): 111-124.

Bigelow – National Center for Marine Algae and Microbiota. https://ncma.bigelow.org/

Bisker, R., and M. Castagna. 1987. Predation on single spat oysters, *Crassostrea virginica* (Gmelin) by blue crabs, *Callinectes sapidus* (Rathburn) and mud crabs, *Panopeus herbstii* (Milne-Edwards). Journal of Shellfish Research 6: 37–40.

Block, W. M. and L. A. Brennan. 1993. The habitat concept in ornithology: Theory and applications. Current Ornithology 11:35-91.

Bonar, D.B., S.L. Coon, M. Walch, R.M. Weiner and W. Fitt. 1990. Control of oyster settlement and metamorphosis by endogenous and exogenous chemical cues. Bulletin of Marine Science 46(2): 484-498.

Boudreaux, M.L., J.L Stiner, L.J. Walters. 2006. Biodiversity of Sessile and Motile Macrofauna on Intertidal Oyster Reefs in Mosquito Lagoon, Florida. Journal of Shellfish Research 25(3): 1079-1089.

Brumbaugh, R.D. and L.D. Coen, 2009. Contemporary approaches for small-scale oyster reef restoration to address substrate versus recruitment limitation: A review and comments relevant for the olympia oyster, *Ostrea lurida* carpenter 1864. Journal of Shellfish Research 28(1): 147-161.

Burke, R.D. 1983. The induction of metamorphosis of marine invertebrate larvae: stimulus and response. Canadian Journal of Zoology 61: 1701-1719.

Burke, R.D. 1986. Pheromones and the gregarious settlement of marine invertebrate larvae. Bulletin of Marine Science 39(2): 323-331.

Burkepile, D.E. and M.E. Hay. 2006. Herbivore vs. nutrient control of marine primary producers: context-dependent effects. Ecology 87(12): 3128-3139.

Caffey, H.M. 1985. Spatial and temporal variation in settlement and recruitment of intertidal barnacles. Ecological Monographs 55(3): 313-332.

Camargo, J.A. and A. Alonso. 2006. Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment. Environmental International 32: 831-849.

Carnegie, R., and E. Burreson. 2011. Declining impact of an introduced pathogen: Haplosporidium nelsoni in the oyster Crassostrea virginica in Chesapeake Bay. Marine Ecology Progress Series 432: 1-15.

CBP- Food Web. Chesapeake Bay Program: Discover the Chesapeake – Food Webs. Acquired from: http://www.chesapeakebay.net/discover/bayecosystem/foodwebs.

Chesapeake Bay Program-Salinity. Map: Chesapeake Bay Mean Surface Salinity- Spring (1985-2006). http://www.chesapeakebay.net/maps/map/chesapeake_bay_mean_surface_salinity_spring_1985_2006

Ciarniello L.M., M.S. Boyce, D.R. Seip, and D.C. Heard. 2007. Grizzly Bear Habitat Selection is Scale Dependent. Ecological Applications 17(5): 1424-1440.

Coen, L.D, R. Brumbaugh, D. Busek, R. Grizzle, M. Luckenbach, M. Posey, S. Powers, and S. Tolley. 2007. Ecosystem services related to oyster restoration. Marine Ecology Progress Series 341:303-301.

Cole, H.A., E.W. Knight-Jones. 1949. The settling behavior of larvae of the European oyster *Ostrea edulis* L. and its influence on methods of cultivation and spat collection. Fisheries Investigation (London) Series 2, 17: 1-39.

Cook, T., M. Folli, J. Klinck, S. Ford, and J. Miller. 1998. The Relationship Between Increasing Sea-surface Temperature and the Northward Spread of Perkinsus marinus (Dermo) Disease Epizootics in Oysters. Estuarine, Coastal, and Shelf Science 46: 587-597.

Coon, S.L. and D.B. Bonar. 1985. Induction of settlement and metamorphosis of the pacific oyster *Crassostrea gigas* (Thunberg), by L-DOPA and catecholamine's. Journal of Experimental Biology 94: 211-221.

Crisp, D. J. 1967. Chemical Factors Inducing Settlement in Crassostrea virginica. Journal of Animal Ecology 36(2): 329-335.

Clayton, J.L., E.S. Dannaway, R. Menendez, H.W. Rauch, J.J. Renton, S.M. Sherlock, and P.E. Zurbuch. 1997. Application of limestone to restore fish communities in acidified streams. North American Journal of Fisheries Management 18(2): 347-360.

Dixson, D.L., G.P. Jones, P.L. Minday, M.S. Pratchett, M. Srinivasan, S. Planes, S.R. Thorrold. 2011. Terrestrial chemical cues help coral reef fish larvae locate settlement habitat surrounding islands. Ecology and Evolution 1(4): 586-595.

Donzár J.A., J.J. Negro and F. Hiraldo. 1993. Foraging habitat selection, land-use changes and population decline in the lesser kestrel *Falco naumanni*. Journal of Applied Ecology. 30: 515-522.

Drexler, M., M. Parker, S. Geiger, W. Arnold, and P. Hallock. 2014. Biological Assessment of Eastern Oysters (*Crassostrea virginica*) Inhabiting Reef, Mangrove, Seawall, and Restoration Substrates. Estuaries and Coasts 37: 962-972.

Dunn, R.P., D.B. Eggleston, and N. Lindquist. 2014. Effects of Substrate Type on Demographic Rates of Eastern Oyster (*Crassostrea virginica*). Journal of Shellfish Research 33: 177-185.

EPA. 2012. 5.9 Conductivity. In Water: Monitoring and Assessment.

Faimali, M., F. Garaventa, A. Terlizzi, M. Chiantore, and R. Cattaneo-Vietti. 2004. The interplay of substrate nature and biofilm formation in regulating *Balanus amphitrite* Darwin, 1854 larval settlement. Journal of Experimental Marine Biology and Ecology 306: 37-50.

Fretwell, S. D. 1972. Populations in Seasonal Environments. Princeton University Press, Princeton, New Jersey.

Fretwell, S. D. and H. L. Lucas Jr.. 1970. On territorial behavior and other factors influencing habitat distributions of birds. I. Acta Biotheoretica 19:16–36

Gobler, C. J., D. L. Berry, S. T. Dyhrman, S. W. Wilhelm, A. Salamov, A. V. Lobanov, Y. Zhang, J. L. Collier, L. L. Wurch, A. B. Kustka, B. D. Dill, M. Shah, N. C. VerBerkmoes, A. Kuo, A. Terry, J. Pangilinan, E. A. Lindquist, S. Lucas, I. T. Paulsen, T. K. Hattenrath-Lehmann, S. C. Talmage, E. A. Walker, F. Koch, A. M. Burson, M. A. Marcoval, Y.Z. Tang, G. R. LeCleir, K. J. Coyne, G. M. Berg, E. M. Bertrand, M. A. Saito, V. N. Gladyshev, I. Grigoriev, 2011. Niche of harmful alga *Aureococcus anophagefferens* revealed through ecogenomics. Proceedings of the National Academy of Sciences of the United States of America, 108(11), 4352–4357.

Gottlieb S.J. and M.E. Schweighofer. 1996. Oysters and the Chesapeake Bay Ecosystem: A Case for Exotic Species Introduction to Improve Environmental Quality? Estuaries 19(3): 639-650.

Grabowski, J.H. 2004. Habitat complexity disrupts predator-prey interactions but not the trophic cascade on oyster reefs. Ecology 85: 995-1004.

Grabowski, J.H. and Peterson, C.H., 2007. Restoring oyster reefs to recover ecosystem services. Theoretical ecology series 4:281-298.

Guillard, R.R.L. and Hargraves, P.E. 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. Phycologia 32: 234-236.

Gutiérrez, J. L., C. G. Jones,D. L. Strayer, and O. O. Iribarne. 2003. Mollusks as ecosystem engineers: the role of shell production in aquatic habitats. Oikos101: 79–90.

Hamester, M.R.R., P.S. Balzer, and D. Becker. 2012. Characterization of calcium carbonate obtained from oyster and mussel shells and incorporation in polypropylene. Materials Research 15(2): 204-208.

Hastie G.D., B. Wilson, P.M. Thompson. 2003. Fine-scale habitat selection by coastal bottlenose dolphins: applications of a new land based video-montage technique. Canadian Journal of Zoology 81: 469-478.

Hidu, H. 1969. Gregarious Setting in the American Oyster *Crassostrea virginica* (Gmelin). Chesapeake Science 10 (2): 85-92.

Hidu,H., S.Chapman, and P.W.Soule. 1975. Cultchless setting of Europeanoysters, *Ostrea edulis*, using polished marble. Proceedings of the National Shellfisheries Association 65: 13–14.

Holligan, P. M. , E. Fernández, J. Aiken, W. M. Balch, P. Boyd, P. H. Burkill, M. Finch, S. B. Groom, G. Malin, K. Muller, D. A. Purdie, C. Robinson, C. C. Trees, S. M. Turner, P. van der Wal. 1993. A biogeochemical study of the coccolithophore, *Emiliania huxleyi*, in the North Atlantic. Global Biogeochemical Cycles 7 (4): 879-900.

Hudy, M., D.M. Downey, and D.W. Bowman. 2000. Successful restoration of an acidified native brook trout stream through mitigation with limestone sand. North American Journal of Fisheries Management 20(2): 453-466.

Hutto, R.L. 1985. Habitat Selection by Nonbreeding, Migratory Land Birds. Pages 455-476 in Habitat Selection in Birds (M.L. Cody, Ed.) Academic Press, New York.

Iglesias-Rodrigues, M.D., P.R. Halloran, R.E. Rickaby, I.R. Hall, E. Colmenero-Hidalgo, J.R. Gittins, D.R. Green, T.Tyrrell, S.J. Gibbs, P. von Dassow, E. Rehm, E.V. Armbrust, and K.P. Boessenkool. 2008. Phytoplankton calcification in a high-CO2 world. Science 320(5874): 336-340.

Jacksonville Shell Club. Found at http://www.jaxshells.org/936.htm.

James Cook University- Tropical Water & Aquatic Ecosystem Research. Found at https://research.jcu.edu.au/tropwater/research-programs/coastal-estuarine-ecology/shellfish-reef-protection-and-repair/what-are-shellfish-reefs.

Jones J. 2001. Habitat Selection Studies in Avian Ecology: A Critical Review. The Auk 118: 556-562.

Kannan, K., K.J. Hansen, T.L. Wade, and J.P Giesy. 2002. Perfluorooctane sulfonate in osyters, *Crassostrea virginica,* from the Gulf of Mexico and the Chesapeake Bay, USA. Archives of Environmental Contamination and Toxicology 42: 313-318.

Kaushal, S.S., G.E. Likens, R.M. Utz, M.L. Pace, M.Grese, and M. Yepsen. 2013. Increased river alkalinization in the eastern U.S. Environmental Science and Technology 47: 10302-10311.

Kellogg M. L., J.C. Cornwell, M.S. Owens, and K.T. Paynter. Denitrification and Nutrient Assimilation on a Restored Oyster Reef. Marine Ecology Progress Series 480: 1-19.

Kemp, W., W. Boynton, J. Adolf, D. Boesch, W. Boicourt, G. Brush, J. Cornwell, T. Fisher, P. Glibert, J. Hagy, L. Harding, E. Houde, D. Kimmel, W. Miller, R. Newell, M. Roman, E. Smith, and J. Stevenson. 2005. Eutrophication of Chesapeake Bay: historical trends and ecological interactions. Marine Ecology Progress Series 303: 1-29.

Kendeigh, S.C. 1945. Community selection in birds on the Heldenberg Plateau of New York. Auk 62:418-436.

Koch, M., G. Bowes, C. Ross, and X. Zhang. 2012. Climate change and ocean acidification effects on seagrasses and marine macroalgae. Global Climate Change 19(1): 103-132.

Krantz, G. E. and J. V. Chamberlin. 1978. Blue crab predation on cultchless oyster spat. Proceedings of the National Shellfisheries Association 68: 38–41.

Krienitz, L. and M. Wirth. 2006. The high content of polyunsaturated fatty acids in *Nannochloropsis limnetica* (Eustigmatophyceae) and its implication for food web interactions, freshwater aquaculture and biotechnology. Limnologica – Ecology and Management of Inland Waters 36(3): 204-210.

Kroeker, K.J., R.L. Kordas, R. Crim, I.E. Hendriks, L. Romajo, G.S. Singh, C.M. Duarte, and J. Gattuso. 2013. Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming. Global Change Biology 19(6): 1884-1896.

Kurihara, H. and A. Ishimatsu, 2007. Effects of Increased Seawater pCO2 on Early Development of the Oyster *Crassostrea gigas*. Aquatic Biology 1: 91-98.

Land, E.D., D.B. Shindle, R.J. Kawula, J.F. Benson, M.A. Lotz, D.P. Onorato. 2008. Florida Panther Habitat Selection Analysis of Concurrent GPS and VHF Telemetry Data. The Journal of Wildlife Management 72(3): 633-639.

Lecchini, D., D.L. Dixson, G. Lecellier, N. Roux, B. Frédérich, M. Besson, Y. Tanaka, B. Banaigs, Y. Nakamura. 2017. Habitat selection by marine larvae in changing chemical environments. Marine Pollution Bulletin 114(1): 210-217.

Lecchini, D., S.C. Mills, C. Brié, R. Maurin, B. Banaigs. 2010. Ecological determinants and sensory mechanisms in habitat selection of crustacean postlarvae. Behavioral Ecology 21(3): 599-607.

Lehotay, S.J., J.A. Harman-Fetcho, and L.L McConnell. 1998. Agricultural pesticide residues in oysters and water from two Chesapeake Bay tributaries. Marine Pollution Bulletin 37(1-2): 32-44.

Liu, X. and B. Huang. 2000. Heat stress injury in relation to membrane lipid peroxidation in creeping bentgrass. Crop Science 40: 503-510.

Nair, M.R. and K.K. Appukuttan. 2003. Effect of temperature on the development, growth, survival and settlement of green mussel *Perna viridis* (Linnaeus, 1758). Aquaculture Research 34: 1037-1045.

Mann, R., Powell, E.R. 2007. Why oyster restoration goals in the Chesapeake Bay are not and probably cannot be achieved. Journal of Shellfish Research. 26(4):905-917.

Mann R., M.Southworth, J.M. Harding, J. A. Wesson. 2009. Population Studies of the Native Eastern Oyster, *Crassostrea virginica*, (Gmelin, 1791) in the James River, Virginia, USA. Journal of Shellfish Research 28:193-220.

Mather, B. 1964. Effects of seawater on concrete. U.S. Army Engineer Waterways Experiment Station: Corps of Engineers.

Maracoos: Mid-Atlantic Regional Association Coastal Ocean Observing System. 1993-2014 Temperature Map. http://maracoos.org/node/455.

Mauritzen, M., S. E. Belikov, A. N. Boltunov, A. E. Derocher, E. Hansen, R. A. Ims, 0. Wiig, and N. Yoccoz. 2003. Functional responses in polar bear habitat selection. Oikos 100: 112-124.

Mayer, P., R. Cuhel, and N. Nyholm. 1997. A simple *in vitro* fluorescence method for biomass measurements in algal growth inhibition tests. Water Research 31(10): 2525-2531.

McDermott, J. J. 1960. The predation of oysters and barnacles by crabs of the family Xanthidae. Proceedings of the Pennsylvania Academy of Science 354: 199–211.

McVey, J.P. 1970. Fishery ecology of the Pokai artificial reef. Ph.D. Dissertation, University of Hawaii, Honolulu.

Meadows P.S. and J.I. Campbell. 1972. Habitat Selection by Aquatic Invertebrates. Advances in Marine Biology 10: 271-382.

Meer, van der J., J.J., Beukema, and R. Dekker. 2002. Large spatial variability in lifetime egg production in an intertidal baltic tellin (*Macoma balthica*) population. Helgoland Marine Research (2003) 56: 274–278.

Menendez, R., J.L. Clayton, and P.E. Zurbuch. 1996. Chemical and fishery responses to mitigative liming of an acidic stream, Dogway Fork, West Virginia. Restoration Ecology 4(3): 220-233.

Menge, B.A. 1992. Community regulation: Under what conditions are bottom-up factors important on rocky shores? Ecology 73(3): 755-765.

Miller, R.L., W.L. Bradford, and N.E. Peters. 1988. Specific Conductance: Theoretical Considerations and Application to Analytical Quality Control. In: U.S.G.S. Water-Supply Paper.

Morrissey J.F. and S.H. Gruber. 1993. Habitat selection by juvenile lemon sharks, *Negaprion brevirostris*. Environmental Biology of Fishes 38: 311-319.

Moss, K.R.K and S.M. Moss. 2004. Effects of artificial substrate and stocking density on the nursery production of pacific white shrimp *Litopenaeus vannamei.* Journal of the World Aquaculture Society 35(4): 536-542.

Muthiah, P., D. Sundararajan, G. Srinivasan, and N. Vaithianathan. 1987. Pests and predators of oysters. Central Marine Fisheries Research Institute: Oyster Culture – Status and Prospectus 38: 71-74.

NERI. National Estuaries Restoration Inventory. https://neri.noaa.gov/neri/.

Nestlerode J.A., M.W.Luckenbach, and F.X.O'Beirn. 2007. Settlement and Survival of the Oyster *Crassostrea virginica* on Created Oyster Reef Habitats in Chesapeake Bay. Restoration Ecology 15(2): 273-283.

Ocean Cove Seafood. Restoring the Bay: Decline of Oysters in the Chesapeake Bay. Found at http://oceancoveclams.com/restoring-the-environment/.

Orr J.C., V.J. Fabry, O. Aumont, L. Bopp, S.C. Doney, R.A. Feely, A. Gnanadesikan, N.Gruber, A. Ishida, F. Joos, R. M. Key, K. Lindsay, E. Maier-Reimer, R. Matear, P. Monfray, A.Mouchet, R.G. Najjar, G. Plattner, K.B. Rodgers, C.L. Sabine, J.L. Sarmiento, R.Schlitzer, R.D. Slater, I.J. Totterdell, M.Weirig, Y. Yamanaka, and A.Yool. 2005. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature 437: 681-686.

Pace, M.L., J.J. Cole, S.R. Carpenter, and J.F. Kitchell. 1999. Trophic cascades in diverse ecosystems. Trends in Ecology and Evolution 14(12): 483-488.

Paine, R.T., 1980. Food webs: linkage, interaction strength and community infrastructure. Journal of Animal Ecology 49: 667-685.

Palenik, B., J. Grimwood, A. Aerts, P. Rouz, A. Salamov, N. Putnam, C. Dupont, R. Jorgensen, E. Derelle, S. Rombauts, K. Zhou, R. Otillar, S. S. Merchant, S. Podell, T. Gaasterland, C. Napoli, K. Gendler, A. Manuell, V. Tai, O. Vallon, G. Piganeau, S. Jancek, M. Heijde, K. Jabbari, C. Bowler, M. Lohr, S. Robbens, G. Werner, I. Dubchak, G. J. Pazour, Q. Ren, I. Paulsen, C. Delwiche, J. Schmutz, D. Rokhsar, Y. Van de Peer, H. Moreau, and I. V. Grigoriev. 2007. The tiny eukaryote *Ostreococcus* provides genomic insights into the paradox of plankton speciation. Proceedings of the National Academy of Sciences 104:7705-7710.

Parkhill, J.P., G. Maillet, J.J. Cullen. 2001. Fluorescence-based maximal quantum yield for PSII as a diagnostic of nutrient stress. Journal of Phycology 37(4): 517-529.

Peterson, C., J. Grabowski, and S. Powers. 2003. Estimated enhancement of fish production resulting from restoring oyster reef habitat: quantitative valuation. Marine Ecology Progress Series 264: 249-264.

Pond, D.W., R.P. Harris. 1996. The lipid composition of the coccolithophore *Emiliania huxleyi* and its possible ecophysiological significance. Journal of the Marine Biological Association of the United Kingdom 76(3): 579-594.

Porzio, L., M.C. Buia, and J.M. Hall-Spencer. 2011. Effects of ocean acidification on microalgal communities. Journal of Experimental Marine Biology and Ecology 400: 278-287.

Raimondi, P.T. 1988. Settlement cues and determination of the vertical limit of an intertidal barnacle. Ecology 69(2): 400-407.

Reef Innovations. Found at http://reefinnovations.com/.

Riebesell, U., I. Zondervan, B. Rost, P.D. Tortell, R.E. Zeebe, and F.M.M. Morel. 2000. Reduced calcification of marine plankton in response to increased atmospheric CO2. Letters to Nature 407: 364-367.

Roberts, D., D. Rittschof, E. Holm, and A.R. Schmidt. 1991. Factors influencing initial larval settlement: temporal, spatial and surface molecular components. Journal of Experimental Marine Biology and Ecology. 150 (1991): 203-211.

Rothschild, B., J. Ault, P. Goulletquer, and M. Héral. 1994. Decline of the Chesapeake Bay oyster population: a century of habitat destruction and overfishing. Marine Ecology Progress Series 111:29-39.

Schulte, D.M. and R.P. Burke. 2014. Recruitment Enhancement as an Indicator of Oyster Restoration Success in Chesapeake Bay. Ecological Restoration 32(4): 434-440.

Schulte, D.M., R.P. Burke, and R.N. Lipcius. 2009. Unprecedented Restoration of a Native Oyster Metapopulation. Science 325: 1124-1128.

Scheitzer, R., R. Arantes, M.F. Baloi, P.F.S. Costodio, L.V. Arana, W.Q. Seiffert, and E.R. Andreatta. 2013. Use of artificial substrates in the culture of *Litopenaeus vannamei* (Biofloc System) at different stocking densities: Effects on microbial activity, ewater quality, and production rates. Aquaculture Engineering 54: 93-103.

Shi, D., Y. Xu, and F.M.M. Morel. 2008. Effects of the pH.pCO2 control method on medium chemistry and phytoplankton growth. Biogeosciences 6: 1199-1207.

Sindermann, C.J. 1995. Ocean pollution: effects on living resources and humans. CRC Marine Science. CRC Press.

Soniat T.M. and G.M. Burton. 2005. A Comparison of the Effectiveness of Sandstone and Limestone as Cultch for Oysters, *Crassostrea virginica*. Journal of Shellfish Research 24: 483-485.

Soniat T.M., R.C. Broadhurst, and E.L.Haywood.1991. Alternatives to clam shell as cultch for oysters, and the use of gypsum for the production of cultchless oysters. Journal of Shellfish Research 10: 405–410.

South Carolina Department of Natural Resources. Sea Science: Blue Crab. Found at http://www.dnr.sc.gov/marine/pub/seascience/bluecrab.html.

Stepien, P. and G. Klobus. 2006. Water relations and photosynthesis in *Cucumis sativus* L. leaves under salt stress. Biologia Plantarum 50(4): 610-616.

Svaärdson, G. 1949. Competition and habitat selection in birds. Oikos 1:157-174.

Talmage, S. C., and C. J. Gobler. 2009. The effects of elevated carbon dioxide concentrations on the metamorphosis, size, and survival of larval hard clams (Mercenaria mercenaria), bay scallops (Argopecten irradians), and Eastern oysters (Crassostrea virginica). Limnology and Oceanography 54:2072-2080.

Tamburri, M.N., M.W. Luckenbach, D.L. Breitburg, and S.M. Bonniwell. 2008. Settlement of *Crassostrea ariakensis* larvae: Effects of substrate, biofilms, sediment, and adult chemical cues. Journal of Shellfish Research 27(3): 601-608.

Tamburri, M.N., R.K. Zimmer, and C.A. Zimmer. 2007. Mechanisms reconciling gregarious larval settlement with adult cannibalism. Ecological Monographs 72: 255-268.

Tamburri, M.N., R.K. Zimmer-Faust and M.L. Tamplin. 1992. Natural Sources and Properties of Chemical Inducers Mediating Settlement of Oyster Larvae: A Re-examination. Biological Bulletin 183: 327-338.

Theuerkauf S.J., R.P. Burke, and R.N. Lipcius. 2015. Settlement, growth, and survival of eastern oysters on alternative reef substrates. Journal of Shellfish Research. 34(2): 241-272.

Turner, E.J., R. K. Zimmer-Faust, M. A. Palmer, M. Luckenbach, and N. D. Pentcheff. 1994. Settlement of Oyster (*Crassostrea virginica*) Larvae: Effects of Water Flow and a Water-Soluble Chemical Cue. Limnology and Oceanography 39: 1579-1593.

UK Ocean Acidification Research Programme. Found at http://www.oceanacidification.org.uk/.

U.S. Fish and Wildlife Service- Building Living Reefs in Southern New Jersey. Found at https://usfwsnortheast.wordpress.com/2015/05/22/shells-and-students-in-southern-new-jersey/.

Veitch, F.P. and H. Hidu. 1971. Gregarious setting in the American oysters *Crassostrea* virginica (Gmelin): I. Properties of a partially purified "setting factor". Chesapeake Science 12(3): 173-178.

Waldbusser, G.G., E.P. Voigt, H. Bergschneider, M.A. Green, and R.I.E. Newell. 2011. Biocalcification in the Easter Oyster (*Crassostrea virginica)* in relation to long-term trends in Chesapeake Bay pH. Estuaries and Coasts 34: 221-231.

Waldbusser, G.G., R.A. Steenson, and M.A. Green. 2011. Oyster shell dissolution rates in estuarine waters: effects of pH and shell legacy. Journal of Shellfish Research 30(3): 659-669.

Ware, D.M. and R.E. Thomson. 2005. Bottom-up ecosystem trophic dynamics determine fish production in the Northeast Pacific. Science 308: 1280-1284.

Whitman E.R. and M.A. Reidenbach. 2012. Benthic flow environments affect recruitment of *Crassostrea virginica* larvae to an intertidal oyster reef. Marine Ecology Progress Series 463: 177-191.

Wilkie, E.M., M.J. Bishop and W.A. O'Connor. 2013. The density and spatial arrangement of the invasive oyster *Crassostrea gigas* determines its impact on settlement of native oyster larvae. Ecology and Evolution 3(15): 4851-4860.

WHO: World Health Organization. 1996. Water Quality Assessments – A guide to use of biota, sediments, and water in environmental monitoring – Second edition.

Wood, A.M., R.C. Everroad, and L.M. Wingard. 2005. Algal Culturing Techniques: Chapter 18 – Measuring growth rates in microalgal cultures 269-285.

Woodin, S.A. 1991. Recruitment of Infauna: Positive or Negative Cues? American Zoology 31: 797-807.

Woods H., W.J. Hargis Jr., C.H. Hershiner, and P. Mason. 2005. Disappearance of the Natural Emergent 3-Dimensional Oyster Reef System of the James River, Virginia, 1871–1948. Journal of Shellfish Research 24: 139-142.

Yoon, G.L., B.T. Kim, B.O. Kim, and S.H. Han. 2003. Chemical-mechanical characteristics of crushed oyster shell. Waste Management 23: 825-834.

Zimmer‐Faust, R. K. and M. N. Tamburri. 1994. Chemical identity and ecological implications of a waterborne, larval settlement cue. Limnology and Oceanography 39: 1075-1087.

Zu Ermgassen P.S.E., M.D. Spalding, B. Blake, L.D. Coen, B. Dumbauld, S. Geiger, J.H. Grabowski, R. Grizzle, M. Luckenbach, K. McGraw, W. Rodney, J.L. Ruesnik, S.P. Powers, R. Brumbaugh. 2012. Historical ecology with real numbers: past and present extent and biomass of an imperiled estuarine habitat. The Royal Society 279: 3393-3400.